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ML-236B Biosynthesis-Associated DNA

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ML-236B BIOSYNTHESIS-ASSOCIATED DNA

[ML-236B seibutsu-tokeigaku dantei DNA]

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For an explanation of 2—letter codes and other abbreviations, see the section, "Notes on codes and abbreviations" at the beginning of every regular issue of the PCT Gazette.

Technological Field /1*

The present invention relates to DNA characterized by improving ML-236B biosynthesis in microorganisms that produce the HMG-CoA reductase inhibitor ML-236B, nucleic acid molecules that hybridize with said DNA, a recombinant DNA vector that incorporates said DNA, host cells that have been transformed with said recombinant DNA, a ML-236B manufacturing method, and PCR primers designed based on the base sequence of said DNA.

Prior art

Pravastatin is an HMG-CoA reductase inhibitor that has been clinically used as a hyperlipidemia therapeutic, and is obtained by subjecting ML-236B produced by *Penicillium citrinum* to modification using the microorganism *Streptomyces carbophilus* (Endo, A., et al., J. Antibiot., 29, 1346 (1976); Matsuoka, S. et al. Eur. J. Biochem. 184, 707 (1989).

It has also been shown that the HMG-CoA inhibitory lovastatin that shares part of its structure with pravastatin and ML-236B pravastatin precursor is biosynthesized via polyketide (Moore, R.N, et al., J. Am. Chem. Soc. 107, 3694 (1985); Shiao, M. and Don, H.S. Proc. Natl. Sci. Counc. ROC. 11, 223 (1987)).

"Polyketide" is a general term for compounds derived from β-keto carbon chains generated by a continuous condensation reaction between low-molecular-weight carboxylic acid residues such as acetic acid, propionic acid and butyric acid. Various structures are generated, depending on the mode of condensation and reduction for each β-ketocarbonyl group (Hopwood, D. A. and Sherman, D.H., Annu. Rev. Genet. 24, 37-66 (1990); Hutchinson, C.R. and Fujii, I., Annu. Rev. Genet. 49, 201-238 (1995)).

Polyketide synthase (PKS below) which supports polyketide synthesis is known to be an enzyme possessed by fungi and bacteria, and biomolecular research has been carried out on this enzyme in fungi (Feng, G.H. and Leonard, T.J., J. Bacteriol. 177, 6246 (1995); Takano, Y. et al. /2
Mol. Gen. Genet. 249, 162 (1995)). The triol PKS gene has been analyzed in *Aspergillus terreus*, which is a lovastatin-producing microorganism (Japanese National Publication (Kohyo) No. Hei 9[1997]-504436).

In this connection, biosynthesis-associated genes for secondary metabolic products often form genomic clusters in fungi, and the existence of gene clusters that participate in polyketide biosynthesis systems is known. Regarding the biosynthesis of aflatoxin, which is a polyketide

* [Numbers in the margin represent the pagination of the original foreign text.]

generated by *Aspergillus flavus* and *Aspergillus parasiticus*, it is known that cluster structures are formed by genes encoding enzyme proteins that participate in the biosynthesis of PKS and other polyketides, and comparative genomic analysis has been carried out regarding aflatoxin biosynthesis-associated genes in the two microorganisms (Yu, J. et al., Appl. Environ. Microbiol. 61, 2365 (1995)). In the biosynthesis of sterigmatocystin produced by *Aspergillus nidulans*, it has been reported that the biosynthesis-associated genes form a cluster structure in a continuous region of approximately 60 kb on the genome (Brown, D.W. et al., Proc. Natl. Acad. Sci. USA 93, 1418 (1996)).

There is currently insufficient biomolecular research regarding ML-236B biosynthesis.

Disclosure of the Invention

The inventors of the present invention et al. cloned a gene or gene cluster for enzymes that support ML-236B biosynthesis in *Penicillium citrinum* using a genomic DNA library from a microorganism that produces ML-236B, and arrived at the present invention upon discovering that ML-236B biosynthesis is improved when said producing microorganism is transformed with the resulting recombinant DNA vector.

The present invention is:

1) DNA characterized by comprising a base sequence represented by nucleotides 1-34203
of Sequence No. 1 of the sequence table, and by improving ML-236B biosynthesis in ML-236B- /3
producing microorganisms when introduced into said microorganisms;

(2) The DNA recited in (1), which can be obtained from transformed *E. coli* strain pML48 SANK71199;

(3) DNA characterized by hybridizing with the DNA recited in (1) or (2), and by improving ML-236B biosynthesis in ML-236B-producing microorganisms when introduced into said microorganisms;

(4) DNA characterized by hybridizing under stringent conditions with the DNA recited in (1) or (2), and by improving ML-236B biosynthesis in ML-236B-producing microorganisms when introduced into said microorganisms;

(5) Recombinant DNA vector comprising the DNA recited in any one of sections (1)-(4);

(6) The recombinant DNA vector recited in (5) which is carried by the transformed *E. coli* strain pML48 SANK71199 (FERM BP-6780);

(7) Host cells that have been transformed with the recombinant DNA vector recited in (5) or (6);

(8) The host cells recited in (7) characterized by being ML-236B-producing microorganisms;

(9) The host cells recited in (8) characterized by being *Penicillium citrinum*;

(10) A method for manufacturing ML-236B, characterized in that the host cells of (8) or (9) are cultured, and ML-236B is then recovered from said culture;

(11) The host cells according to (7), characterized by being *E. coli*;

(12) The host cells according to (11) which are a transformed *E. coli* strain ML48 SANK71199 (FERM BP-6780);

(13) A PCR primer A1 having a sequence of at least 10 bases wherein its 5'-terminus is the adenine of Nucleotide No. 23045 in Sequence No. 2 of the sequence table, or a base to the 5' side thereof;

(14) A PCR primer A2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of the PCR primer A1 recited in (13) /4
(where said PCR primer A2 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 23045 to 23047 of Sequence No. 2 of the sequence table);

(15) A PCR primer A3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of the PCR primer A1 recited in (13) (where said PCR primer A3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 23045 to 23047 of Sequence No. 2 of the sequence table);

(16) A PCR primer A4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of the PCR primer A1 recited in (13) (where said PCR primer A4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 23045 to 23047 of Sequence No. 2 of the sequence table);

(17) A PCR primer B1 comprising a sequence comprising at least 10 bases wherein its 5'-terminus is the cytosine of Nucleotide No. 1479 in Sequence No. 1 of the sequence table or a base to the 5' side thereof;

(18) A PCR primer B2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer B1 recited in (17) (where said PCR primer B2 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the alanine residue encoded by Nucleotide Nos. 2720 to 32722 of Sequence No. 2 of the sequence table);

(19) A PCR primer B3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer B1 recited in (17) (where said PCR primer B3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the alanine residue encoded by Nucleotide Nos. 2720 to 32722 of Sequence No. 2 of the sequence table);

(20) A PCR primer B4 comprising a sequence comprising at least 10 bases and having at /5
least 90% homology with respect to the base sequence of PCR primer B1 recited in (17) (where said PCR primer B4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the alanine residue encoded by Nucleotide Nos. 2720 to 32722 of Sequence No. 2 of the sequence table);

(21) A PCR primer C1 having a sequence comprising at least 10 based wherein the 5'-terminus is the adenine of Nucleotide No. 11748 in Sequence No. 2 of the sequence table, or a base on the 5'-side thereof;

(22) A PCR primer C2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer C1 recited in (21) (where said PCR primer C2 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 11748 to 11750 of Sequence No. 2 of the sequence table);

(23) A PCR primer C3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer C1 recited in (21) (where said PCR primer C3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 11748 to 11750 of Sequence No. 2 of the sequence table);

(24) A PCR primer C4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer C1 recited in (21) (where said PCR primer C4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 11748 to 11750 of Sequence No. 2 of the sequence table);

(25) A PCR primer D1 having a sequence comprising at least 10 bases, wherein the 5'-terminus is the thymine of Nucleotide No. 14362 in Sequence No. 1 of the sequence table or a base on the 5'-side thereof;

(26) A PCR primer D2 comprising a sequence comprising at least 10 bases and having at
least 70% homology with respect to the base sequence of PCR primer D1 recited in (25) (where /6
said PCR primer D2 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the serine residue encoded by Nucleotide Nos. 19837 to 19839 of Sequence No. 2 of the sequence table);

(27) A PCR primer D3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer D1 recited in (25) (where said PCR primer D3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the serine residue encoded by Nucleotide Nos. 19837 to 19839 of Sequence No. 2 of the sequence table);

(28) A PCR primer D4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer D1 recited in (25) (where said PCR primer D4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the serine residue encoded by Nucleotide Nos. 19837 to 19839 of Sequence No. 2 of the sequence table);

(29) A PCR primer E1 having a sequence comprising at least 10 bases, wherein the 5'-terminus is the adenine of Nucleotide No. 11796 in Sequence No. 1 of the sequence table or a base on the 5'-side thereof;

(30) A PCR primer E2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer E1 recited in (29) (where said PCR primer E2 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 11796 to 11798 of Sequence No. 1 of the sequence table);

(31) A PCR primer E3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer E1 recited in (29) (where said PCR primer E3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 11796 to 11798 of Sequence No. 1 of the sequence table);

(32) A PCR primer E4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer E1 recited in (29) (where /7
said PCR primer E4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 11796 to 11798 of Sequence No. 1 of the sequence table);

(33) A PCR primer F1 having a sequence comprising at least 10 bases, wherein the 5'-terminus is the thymine of Nucleotide No. 20723 in Sequence No. 2 of the sequence table or a base on the 5'-side thereof;

(34) A PCR primer F2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer F1 recited in (33) (where said PCR primer F2 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the cysteine residue encoded by Nucleotide Nos. 13476 to 13478 of Sequence No. 1 of the sequence table);

(35) A PCR primer F3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer F1 recited in (33) (where said PCR primer F3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the cysteine residue encoded by Nucleotide Nos. 13476 to 13478 of Sequence No. 1 of the sequence table);

(36) A PCR primer F4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer F1 recited in (33) (where said PCR primer F4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the cysteine residue encoded by Nucleotide Nos. 13476 to 13478 of Sequence No. 1 of the sequence table);

(37) A PCR primer G1 having a sequence comprising at least 10 bases, wherein the 5'-terminus is the adenine of Nucleotide No. 24321 in Sequence No. 1 of the sequence table or a base on the 5'-side thereof;

(38) A PCR primer G2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer G1 recited in (37) (where said PCR primer G2 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 24321 to 24323 of /8
Sequence No. 1 of the sequence table);

(39) A PCR primer G3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer G1 recited in (37) (where said PCR primer G3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 24321 to 24323 of Sequence No. 1 of the sequence table);

(40) A PCR primer G4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer G1 recited in (37) (where said PCR primer G4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 24321 to 24323 of Sequence No. 1 of the sequence table);

(41) A PCR primer H1 having a sequence comprising at least 10 bases, wherein the 5'-terminus is the thymine of Nucleotide No. 6312 in Sequence No. 2 of the sequence table or a base on the 5'-side thereof;

(42) A PCR primer H2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer H1 recited in (41) (where said PCR primer H2 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the arginine residue encoded by Nucleotide Nos. 27887 to 27889 of Sequence No. 1 of the sequence table);

(43) A PCR primer H3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer H1 recited in (41) (where said PCR primer H3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the arginine residue encoded by Nucleotide Nos. 27887 to 27889 of Sequence No. 1 of the sequence table);

(44) A PCR primer H4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer H1 recited in (41) (where said PCR primer H4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the arginine residue encoded by Nucleotide Nos. 27887 to 27889 of /9
Sequence No. 1 of the sequence table);

(45) A PCR primer I1 having a sequence comprising at least 10 bases, wherein the 5'-terminus is the adenine of Nucleotide No. 3545 in Sequence No. 2 of the sequence table or a base on the 5'-side thereof;

(46) A PCR primer I2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer I1 recited in (45) (where said PCR primer I2 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 3545 to 3547 of Sequence No. 2 of the sequence table);

(47) A PCR primer I3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer I1 recited in (45) (where said PCR primer I3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 3545 to 3547 of Sequence No. 2 of the sequence table);

(48) A PCR primer I4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer I1 recited in (45) (where said PCR primer I4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 3545 to 3547 of Sequence No. 2 of the sequence table);

(49) A PCR primer J1 having a sequence comprising at least 10 bases, wherein the 5'-terminus is the thymine of Nucleotide No. 28472 in Sequence No. 1 of the sequence table or a base on the 5'-side thereof;

(50) A PCR primer J2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer J1 recited in (49) (where said PCR primer J2 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the alanine residue encoded by Nucleotide Nos. 5727 to 5729 of Sequence No. 2 of the sequence table);

(51) A PCR primer J3 comprising a sequence comprising at least 10 bases and having at /10
least 80% homology with respect to the base sequence of PCR primer J1 recited in (49) (where said PCR primer J3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the alanine residue encoded by Nucleotide Nos. 5727 to 5729 of Sequence No. 2 of the sequence table);

(52) A PCR primer J4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer J1 recited in (49) (where said PCR primer J4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the alanine residue encoded by Nucleotide Nos. 5727 to 5729 of Sequence No. 2 of the sequence table);

(53) A PCR primer K1 having a sequence comprising at least 10 bases, wherein the 5'-terminus is the adenine of Nucleotide No. 400 in Sequence No. 2 of the sequence table or a base on the 5'-side thereof;

(54) A PCR primer K2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer K1 recited in (53) (where said PCR primer K2 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 400 to 402 of Sequence No. 2 of the sequence table);

(55) A PCR primer K3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer K1 recited in (53) (where said PCR primer K3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 400 to 402 of Sequence No. 2 of the sequence table);

(56) A PCR primer K4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer K1 recited in (53) (where said PCR primer K4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 400 to 402 of Sequence No. 2 of the sequence table);

(57) A PCR primer L1 having a sequence comprising at least 10 bases, wherein the /11
5'-terminus is the cytosine of Nucleotide No. 32287 in Sequence No. 1 of the sequence table or a base on the 5'-side thereof;

(58) A PCR primer L2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer L1 recited in (57) (where said PCR primer L2 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the alanine residue encoded by Nucleotide Nos. 1912 to 1914 of Sequence No. 2 of the sequence table);

(59) A PCR primer L3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer L1 recited in (57) (where said PCR primer L3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the alanine residue encoded by Nucleotide Nos. 1912 to 1914 of Sequence No. 2 of the sequence table); and

(60) A PCR primer L4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer L1 recited in (57) (where said PCR primer L4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the alanine residue encoded by Nucleotide Nos. 1912 to 1914 of Sequence No. 2 of the sequence table).

The present invention relates to DNA derived from the genome of ML-236B-producing microorganisms (referred to below as "ML-236B biosynthesis-associated DNA"), which is characterized in that ML-236B production capacity is improved by introducing the DNA into said producing microorganisms.

In the present invention, the term "ML-236B-producing microorganism" denotes a microorganism having antecedent natural capacity for biosynthesizing ML-236B. Examples of ML-236B-producing microorganisms that may be cited are ML-236B-producing microorganisms of the *Penicillium* genus, such as *Penicillium citrinum*, *Penicillium brevicompactum* (Brown, A.G. et al., J. Chem. Soc. Perkin-1, 1165 (1976)), and *Penicillium cyclopium* (Doss, S.L. et al., J. /12
Natl. Prod. 49, 357 (1986)). In addition, other examples that may be cited include *Eupenicillium* sp. M6603 (Endo, A., et al., J. Antibiot. Tokyo 39, 1609 (1986)), *Paecilomyces viridis* FERM P-6236; Japanese Kokai Patent Application No. Sho 58[1983]-98092), *Paecilomyces* sp. M2016 (Endo, A. et al., J. Antibiot. Tokyo 39, 1609 (1986)), *Trichoderma longibrachiatum* M6735 (Endo, A. et al., J. Antibiot. Tokyo 39, 1609 (1986)), *Hypomyces chrysospermus* IFO 7798 (Endo, A. et al., J. Antibiot. Tokyo, 39, 1609 (1986)), *Gliocladium* sp. YJ-9515 (WO9806867), *Trichoderma viride* IFO 5836 (Japanese Kokoku Patent No. Sho 62[1987]-1915), and *Eupenicillium reticulisporum* IFO 9022 (Japanese Kokoku Patent No. Sho 62[1987]-19159). Among these ML-236B-producing microorganisms, *Penicillium citrinum* is preferred, and *Penicillium citrinum* strain SANK 13380 is additionally desirable. *Penicillium citrinum* strain SANK 13380 was internationally deposited on December 22, 1992 under Accession No. FERM BP-4129 at the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Japan Ministry of International Trade and Industry (1-3, Tsukuba-shi 1-chome, Ibaraki-ken, Japan).

ML-236B biosynthesis-associated DNA is obtained by using probes designed based on the base sequence of DNA derived from fungi hypothesized to have similar capacity or a genomic DNA library of an ML-236B-producing microorganism.

The method for producing the genomic DNA library has no particular restrictions, provided that it is a common method for producing genomic DNA libraries from fungi. Examples that may be cited include the method of Maniatis et al. (Maniatis, T. et al., Molecular Cloning, A laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989)).

The genomic DNA of the ML-236B-producing microorganism is obtained by recovering cells from a culture of said producing microorganism, physically breaking down the cells, and then extracting and purifying the nuclear DNA. /13

Culturing of ML-236B-producing microorganisms may be carried out under preferred conditions for the specific ML-236B-producing microorganism. For *Penicillium citrinum*, which is the preferred ML-236B-producing microorganism, MBG3-8 medium (composition: 7% (w/v) glycerin, 3% (w/v) glucose, 1% (w/v) soy flour, 1% (w/v) peptone (manufactured by Kyokuto Pharmaceutical Industrial Co.), 1% (w/v) corn steep liquor (Honen Corp.), 0.5% (w/v) sodium nitrate, 0.1% (w/v) magnesium sulfate tetrahydrate, pH 6.5) is inoculated with the cells, and culturing is carried out while shaking and maintaining a temperature of 22-28°C for 3-7 days. This slant is produced by pouring melted PGA agar medium (composition 200 g/L potato extract, 15% (w/v) glycerin, 2% (w/v) agar) into test tubes, and after allowing it to harden while inclined, using a platinum loop in order to inoculate the material with *Penicillium citrinum*, before maintaining a temperature of 22-28°C for 7-15 days. By storing the slant at 0-4°C, it is possible to continually produce microorganism on the slant.

The cells of ML-236-producing microorganism cultured in liquid medium can be collected by centrifuging and cells of this microorganism that have been cultured on solid medium can be collected by a cell separator or other device.

Physical lysis of cells can be carried out by grinding with a mortar and pestle while freezing the cells on liquid nitrogen. Extraction of nuclear DNA from the lysed cells can be carried out using a surfactant such as sodium dodecyl sulfate ("SDS" below). The extracted genomic DNA can be subjected to phenol-chloroform extraction in order to remove protein, and ethanol precipitation can be carried out in order to recovery the material by precipitation.

The resulting genomic DNA is then subjected to restriction digestion using restriction enzymes in order to fragment it. The restriction enzyme used in order to perform restriction digestion has no particular restrictions, provided that the restriction enzyme can be readily procured, and examples that may be cited includes Sau3A1. The fragmented DNA is then /14
subjected to gel electrophoresis and the DNA is recovered from gel containing the genomic DNA of the appropriate size. There are no particular restrictions on the size of the DNA fragments, but 20 kb or greater is preferred.

The DNA vector used for producing the genomic DNA library has no particular restrictions, provided that it has the base sequence necessary for replication in host cells that have been transformed with said DNA vector, and examples that may be cited include plasmid vectors, phage vectors, cosmid vectors and BAC vectors, with cosmid vectors being preferred. In addition, the DNA vector may also be an expression vector. The DNA vector preferably has a

base sequence that conveys selection capacity in the expressed qualities (phenotype) of the host cells that have been transformed with the DNA vector.

The DNA vector preferably is a vector that is appropriate for both cloning and functional expression. The DNA vector is preferably a shuttle vector that can be used for phenotypic transformation in a microorganism group comprising many microorganisms. The shuttle vector has a base sequence that is required for replication in host cells of at least one microorganism group. In addition, it is preferable for the shuttle vector to have a base sequence that confers selection capacity in the respective expressed qualities of multiple microorganism group hosts.

Regarding the construction of a microorganism group that is transformed by the shuttle vector, there are no particular restrictions, provided that one microorganism group is appropriate for cloning and another has ML-236B-production capacity. For example, combinations of microorganisms and fungi or combinations of yeasts and fungi may be cited, with combinations of bacteria and fungi being preferred. Examples of bacteria have no particular restrictions, provided that they are bacteria that are employed in common genetic engineering techniques, and examples that may be cited include *Escherichia* and *Bacillus microorganisms*, with *Escherichia* being preferred and *E. coli* strain XL1-Blue MR being additionally desirable. Examples of yeasts have no particular restrictions, provided that they are yeasts that are used in common genetic engineering techniques. Examples that may be cited include *Saccharomyces cerevisiae*. Examples of fungi include the above ML-236B-producing microorganisms, and the microorganism group of the present invention can thus be selected from among bacteria, fungi and yeasts.

Examples of this type of shuttle vector that may be cited include cosmid vectors having /15
appropriate expression-type selection marker genes and cos sites. An example of a pSAK333 plasmid having an appropriate *E. coli* hygromycin B phosphotransferase gene sequence (Japanese Kokai Patent Application No. Hei 3[1991]-262486) that may be cited is pSAKcos1 produced by inserting the cos site present in cosmid vector pWE15 (Stratagene), but examples are not restricted to this. The procedure for constructing pSAKcos1 is described in Figure 1.

The desired genomic DNA library is produced by introducing a shuttle vector ligated with the above ML-236B-producing genomic DNA fragments into host cells. The host cells are preferably *E. coli*, and more preferably *E. coli* strain XL1-BlueMR. When the host cells are *E. coli* cells, introduction is carried out by *in vitro* packaging. In the present invention, the term "transformation" denotes the introduction of exogenous DNA by means of *in vitro* packaging, and cells containing the exogenous DNA introduced by *in vitro* packaging are included within the meaning of the term "transformed cells".

Screening for the desired clones is carried out using antibodies or nucleic acid probes, but preferably by using nucleic acid probes. The nucleic acid probes can be constructed based on the

base sequence of a polyketide biosynthesis-associated gene from a fungus. There are no particular restrictions on this type of gene, provided that the base sequence is well known and provided that its role in polyketide biosynthesis has been confirmed. However, examples that may be cited include the aflatoxin PKS gene of *Aspergillus flavus* or *Aspergillus parasiticus* and the sterigmatocystin PKS gene of *Aspergillus nidulans*.

The nucleic acid probe may be obtained by synthesis of oligonucleotide probes comprising partial base sequences from genomic DNA, constructing oligonucleotide primers and carrying out a polymerase chain reaction using genomic DNA as a template (PCR below; Saiki, R.K. et al., Science 239, 487 (1988)), or by using mRNA as a template, synthesizing cDNA /16
using reverse transcriptase, and then performing PCR (reverse transcription PCR; "RT-PCR" below).

The method for obtaining nucleic acid probes from ML-236B-producing microorganisms by PCR or RT-PCR is described below. Construction of the primer used in PCR or RT-PCR (referred to below as "PCR primer") can be carried out based on a well-known region of a polyketide biosynthesis-associated gene base sequence, and preferably based on the aflatoxin PKS gene of *Aspergillus flavus* or *Aspergillus parasiticus*, or based on the base sequence of the sterigmatocystin PKS gene of *Aspergillus nidulans*. A PCR primer can be constructed by means of deriving the base sequence from amino acids that are highly conserved across species among any one of these PKS amino acid sequences. There are two methods for deriving the base sequence from the amino acid sequence, one of which is a method whereby a single sequence is derived based on considering the codon usage frequency of the host, or a method in which a mixed sequence is derived using degenerate codons (referred to below as "mixed primers"). In the latter case, the degree of degeneracy can be decreased by including hypoxanthine in the base sequence.

In addition, an appropriate base sequence may be added to the 5' terminus of the primer in addition to the base sequence for annealing with the template. There are no particular restrictions on the base sequence of said primer, provided that it can be used in PCR, but examples include a base sequence that is utilized for cloning processes carried out on PCR products. Examples of this type of base sequence that may be cited are restriction enzyme recognition sequences and base sequences that comprise these restriction enzyme recognition sequences.

In addition, in constructing PCR primers, it is preferable for the sum of the number of cytosine bases and guanine bases to be 40-60% of the total number of bases. In addition, it is preferable for the primer to resist self-annealing. Within a group of PCR primers, it is preferable for the two PCR primers to resist annealing.

In addition, there are no particular restrictions on the base numbers of the PCR primers, /17

provided that they are appropriate for PCR, but the lower limit of this range is 10-14 and the upper limit is 40-60, with a preferred range being 14-40.

In addition, the PCR primers are preferably DNA. Examples of nucleotides that constitute the primers which may be cited include deoxyadenosine, deoxycytidine, deoxythymidine, deoxyguanosine, adenosine, cytidine, uridine and guanosine, as well as deoxyinosine and inosine.

In addition, the 5'-position of the nucleotide which is located at the 5'-terminus of the PCR primer may be a hydroxyl group, or may assume a state in which a monophosphate is ester-bonded to the hydroxyl group.

Synthesis of the PCR primers may be carried out by common methods used in nucleic acid synthesis, and for example, may be carried out by the phosphoroamidite method. In this method, it is appropriate to use an automated DNA synthesizer.

The PCR template is genomic DNA from the ML-236B-producing microorganism, but the RT-PCR template is mRNA from ML-236B-producing microorganism. The RT-PCR template may also be full-length RNA rather than mRNA.

The PCR product or RT-PCR product can be cloned by incorporating the PCR product or RT-PCR product into a DNA vector that is suitable for this purpose. There are no particular restrictions on the DNA vector used in cloning, provided that it is a common DNA vector that is used for cloning DNA fragments. In addition, kits that facilitate cloning of PCR products or RT-PCR products are commercially available, and an example of such a kit is the Original TA Cloning Kit (Invitrogen; using pCR2.1 as the DNA vector).

Obtaining the cloned PCR product involves culturing transformed host cells confirmed to contain the desired PCR product, extracting plasmid from said cells, purifying it, and then recovering the inserted DNA fragment from the resulting plasmid.

Culturing of the transformed host cells can be carried out under condition appropriate for /18
the host cells. Culturing of transformants of *E. coli,* which is a preferred host cell, can be carried out by maintaining the cells at 30-37°C while shaking for 18 h to 2 days in LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride).

Preparation of plasmid from culture of transformed host cells is carried out by recovering the host cells and removing the genomic DNA or proteins. Preparation of plasmids from culture of transformants of *E. coli* which is a preferred host cell can be carried out by the alkali technique of Maniatis et al. (Maniatis, T. et al., Molecular Cloning, A laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989)). In addition, kits whereby higher purity plasmids can be obtained are commercially available, and an example of such a kit that is desirable for use is the Plasmid Mini Kit (Qiagen). Kits for preparing large

quantities of plasmid are also commercially available, and an example of such a kit that is desirable for use is the Plasmid Maxi Kit (Qiagen).

The DNA concentration of the resulting plasmid can be calculated by measuring the absorbance at a wavelength of 260 nm after appropriate dilution of the DNA sample, based on the fact that one unit of absorption equals 50 μg/mL DNA. The purity of the DNA can be calculated based on the absorption ratio at a wavelength of 280 nm and a wavelength of 260 nm.

Labeling of nucleic acid probes can be broadly categorized into radioactive labeling and nonradioactive labeling. Radioactive species that are used in radioactive labeling have no particular restrictions, provided that they are commonly used, and examples that may be cited include ^{32}P, ^{35}S and ^{14}C, with ^{32}P being preferred. There are no particular restrictions on reagents used for nonradioactive labeling, provided that they are reagents that are commonly used in labeling nucleic acids. Examples that may be cited include digoxigenin and biotin, with digoxigenin being preferred. There are no particular restrictions on the method for labeling the nucleic acid probes, provided that the method is commonly used. Based on the type of nucleic acid probe and other factors, an appropriate selection may be made, for example, from methods in which the label is incorporated into the product by means of PCR employing labeled substrate, nick-translation methods, random primer methods, terminal labeling methods and methods involving oligo-DNA synthesis using labeled substrate.

The presence of the base sequence of the nucleic acid probe in the genome of the /19
ML-236B-producing microorganism can be determined by Southern blot hybridization using genomic DNA from the producing microorganism.

Southern blot hybridization may be carried out according to the method of Maniatis et al. (Maniatis, T. et al., Molecular Cloning, A laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989)).

The target clones can be screened from genomic DNA library using labeled nucleic acid probe produced in the manner described above. There are no particular restriction on the screening method, provided that it is a common method used in genetic cloning. However, it is preferable to use the colony hybridization method (Maniatis, T. et al., Molecular Cloning, A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989)).

Culturing of colonies used in colony hybridization can be carried out under conditions appropriate for the host cells, and culturing of transformants of *E. coli*, which is an appropriate host, can be carried out by maintaining a temperature of 30-37°C for 18 h to 2 days on LB agar medium (1% (v/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride, 1.5% (w/v) agarose).

Preparation of recombinant DNA vector from positive clones obtained by colony hybridization involves extraction and purification of plasmid from cultures of positive clones.

The transformed *E. coli* strain pML48 SANK71199, a positive colony obtained by means of the present invention, was internationally deposited on July 7, 1999 under Accession No. FERM BP-6780 at the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Japan Ministry of International Trade and Industry (1-3, Tsukuba-shi 1-chome, Ibaraki-ken, Japan). The recombinant DNA vector harboring *E. coli* strain pML48 SANK71199 was named "pML48".

It can be confirmed that the recombinant DNA vector carried by the positive clones /20
contains the desired ML-236B biosynthesis-associated DNA by means of functional expression, Southern blot hybridization or identification of the inserted base sequence in the recombinant DNA vector.

The DNA base sequence can be determined by the Maxim-Gilbert chemical modification method (Maxim, A.M.M. and Gilbert W., methods in Enzymology 65, 499 (1980)) or by the dideoxynucleotide chain termination method (Messing, J. and Vieira, J., Gene 19, 269 (1982)). It is desirable to use high grade product with higher purity as the plasmid DNA that is subjected to base sequence identification.

The pML48 inserted base sequence is shown in Sequence No. 1 of the sequence table. The base sequence presented in Sequence No. 2 of the sequence table is completely complementary with respect to the base sequence presented in Sequence No. 2 [sic; Sequence No. 1]. The genomic DNA base sequences have genetic polymorphism among the same species. In addition, there is a determinant probability of the appearance of nucleotide substitutions, etc. during the DNA cloning process and base sequence identification process. Consequently, the present invention includes ML-236B biosynthesis-associated DNA that hybridizes with DNA having the base sequence represented by Nucleotide Nos. 1 to 34203 of Sequence No. 1 or 2 of the sequence table and ML-236B biosynthesis-associated DNA that hybridizes under stringent conditions with DNA having the base sequence represented by Nucleotide Nos. 1 to 34203 of Sequence Nos. 1 or 2 of the sequence table. These DNA sequences are sequences produced by one or more nucleotide conversions, deletions and/or additions to the base sequence represented by Nucleotide Nos. 1 to 34203 of Sequence No. 1 or 2 of the sequence table, as well as sequences derived from ML-236B-producing microorganisms other than *Penicillium citrinum* SANK13380, and the sequences also have the function of improving ML-236B production capacity of ML-236B-producing microorganisms when introduced into these microorganisms. In the present invention, the term "hybridize" denotes that double-stranded DNA is formed in regions where the two single nucleic acid strands are complementary or regions that have high complementarity, thereby forming a double-stranded nucleic acid. The term "stringent conditions" refers to cases where the temperature is maintained at 55°C as hybridization is

carried out using 6x SSC as the hybridization solution composition (1x SSC composition: 150 mM NaCl, 15 mM trisodium citrate).

Analysis methods used for the ML-236B biosynthesis-associated DNA involve the methods according to (1) to (3) below. /21

1) Analysis using gene analysis software

Estimation of the gene region in the genomic DNA sequence was carried out using an existing genetic analysis program (gene finding program; "GRAIL" below) and sequence homology detection programs (BLASTN and BLASTX).

GRAIL is a program that resolves a genome sequence according to seven parameters for evaluating "gene sequence certainty," and synthesizes these results using a neural net method in order to detect structural genes on the genomic DNA (Uverbacher, E.C. & Mural, R.J. Proc. Natl. Acad. Sci. USA 88, 11261 (1991)). It is appropriate to us the ApoCom GRAIL Toolkit (APOCOM).

Blast is a program that employs an algorithm for detecting amino acid sequence and nucleic acid sequence homology (Altechul, S.F., Madden, T.L., et al., Nucl. Acids. Res. 25, 3389 (1997)).

The genomic DNA sequence is resolved into appropriate lengths and homology with respect to a gene database is detected using BLASTN. By this means, it is possible to estimate the location and direction of a structural gene on a test DNA sequence. In addition, the resolved genomic DNA sequence is translated into amino acid sequences based on six translation frames (three each for the sense sequence and antisense sequence), and then detection of homology of the amino acid sequences with respect to a peptide database is carried out using BLASTX, thereby allowing estimation of the location and orientation of the structural genes on the test DNA sequence. Moreover, there are cases where the coding region of a structural gene contained in a genomic DNA sequence is interrupted by an intron sequence in yeast, and in analyzing structural genes having these types of gaps, it is more effective to use BLAST for sequences containing gaps. Consequently, Gapped-BLAST (BLAST 2; included in Wisconsin GCG package ver. 10.0) is appropriate for use.

2) Analysis using Northern blot hybridization

It is possible to use Northern blot hybridization to investigate expression of hypothesized /22
structural genes by the analysis method described in (1).

The total RNA from the ML-236B-producing microorganism subjected to Northern blotting can be obtained by culturing the microorganism. Culturing of *Penicillium citrinum* which is a suitable ML-236B-producing microorganism is carried out by inoculating MGB3-8

medium with microorganisms from a slant of the microorganisms, and then maintaining the cells at 22-28°C for 1-4 days while shaking.

Extraction of RNA from ML-236B-producing microorganism has no particular restrictions, provided that a method that is commonly used for total RNA preparation is employed. Examples that may be cited include the guanidine thiocyanate-hot phenol method, or guanidine thiocyanate-guanidine hydrochloride method. In addition, an example of a commercially-available kit for preparing total RNA with high purity that may be cited is the RNeasy Plant Mini Kit (Qiagen). mRNA can also be obtained by adding the total RNA to an oligo(dT) column and then recovering the fraction that has adsorbed to the column.

Transfer of RNA onto membrane, probe preparation, hybridization and signal detection can be carried out in the same manner as in Southern hybridization described above.

3) 5'-terminus and 3'-terminus analysis

5'-terminal and 3'-terminal analysis of each structural gene an be carried out by the RACE method (rapid amplification of cDNA ends). RACE is a method wherein mRNA is used as a template, and cDNA comprising a region up to the 3'-terminus or 5'-terminus for which the base sequence has not been determined is obtained by RT-PCR from a region for which the base sequence has been determined (Frohman, M.A. et al., Proc. Natl. Acad. Sci. USA 85, 8998 (1988)).

5'-RACE is carried out by the method described below. mRNA is used as template, and oligoDNA (1) on the antisense side designed based on a known location in the base sequence is used as primer for a reverse transcriptase reaction that synthesizes a cDNA primary chain. A homopolymeric (composed of a single base type) nucletotide chain is then attached to the /23
3'-terminus of the cDNA primary chain by means of terminal deoxynucleotidyl transferase. The cDNA primary chain is then used as a template, and double-stranded cDNA in the 5'-terminal region is amplified by means of PCR using, as primer, antisense oligo-DNA (2) present on the 3' side of the oligo-DNA (1) and sense oligo-DNA comprising a base sequence complementary to the homopolymeric base sequence (Frohman, M.A., Methods in Enzymol. 218, 340 (1993)). Kits for 5'-RACE are commercially available, and an example of a preferred kit of this type is the 5'-RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (GIBCO).

3'-RACE is a method that utilizes the poly-A region present at the 3'-terminus of mRNA. Specifically, mRNA is used as template in a reverse transcriptase reaction using oligo-d(T) adapter as primer, thereby synthesizing a cDNA primary chain, whereupon this cDNA primary chain is used as template in order to amplify double-stranded cDNA in the 3'-terminal region by means of PCR using, as primer, antisense oligo-d(T) adapter and sense oligo-DNA (3) designed based on a known region of the base sequence. Kits for 3'-RACE are commercially available,

and an example of a preferred kit of this type is the Ready-To-Go T-Primed First-Strand Kit (Pharmacia).

In designing primers based on known regions of the base sequences used in RACE, it is desirable to use the results of analysis from (1) and (2) above.

By means of the analytical methods described in sections (1) to (3) above, it is possible to estimate the transcription initiation sites, translation initiation codon locations and translation termination codons positions thereof on the structural genes, as well as the orientations of the structural genes on the genomic DNA sequence. Based on these data, it is possible to obtain various structural genes and the cDNA sequences thereof.

Six structural genes were hypothesized to be present on the recombinant DNA vector pML48 insertion sequence obtained in the present invention , and these genes were named mlcA, mlcB, mlcC, mlcD, mlcE and mlcR. Among these genes, mlcA, mlcB, mlcE and mlcR were conjectured to have coding regions on the base sequence represented by Sequence No. 2 of the sequence table, and mlcC and mlcD were conjectured to have coding regions on the base /24
sequence represented by Sequence No. 1 of the sequence table.

The method for obtaining cDNA that may be cited involves carrying out cloning by means of RT-PCR using primers designed based on the above data or cloning from a cDNA library obtained using DNA probes based on these data.

In order to effect functional expression of cDNA that can be obtained by these methods, it is necessary to obtain full-length cDNA. In addition, in order to obtain cDNA that can be functionally expressed by RT-PCR, it is necessary for the RT-PCR product to contain a transcription initiation codon at the original position and to design primers so that there is no translation termination codon at positions other than the original position in the translation frame starting at this translation initiation codon.

PCR primers X1 (where X is selected from A, C, E, G, I and K, and Al is described in (13), C1 is described in (21), E1 is described in (29), G1 is described in (37), I1 is described in (45) and K1 is described in (53)), or PCR primers Y1 (Y is selected from B, D, F, H, J or L and B1 is described in (17), D1 is described in (25), F1 is described in (33), H1 is described in (41), J1 is described in (49) and L1 is described in (57)) have base sequences comprising at least 10 bases in the base sequence presented in Sequence No. 1 or Sequence No. 2 of the sequence table.

The base sequences present in the PCR primers anneal selectively with the template strand, and provided that they can function as primers in PCR or RT-PCR, they need not be completely complementary with respect to a region of the template strand.

The PCR primers X2 through X4 (X is selected from any of A, C, E, G, I and K, A2 is described in (14), A3 is described in (15), A4 is described in (16), C2 is described in (22), C3 is described in (23), C4 is described in (24), E2 is described in (30), E3 is described in (31), E4 is

described in (32), G2 is described in (38), G3 is described in (39), G4 is described in (40), I2 is described in (46), I3 is described in (47), I4 is described in (48), K2 is described in (54), K3 is described in (55), K4 is described in (56)) have a homology of 70% or greater with respect to the base sequence of PCR primer X1 (X in X1 and X2-X4 denotes the group represented by the letter of the alphabet; where A2-A4 correspond to A1, C2-C4 correspond to C1, E2-E4 correspond to E1, G2-G4 correspond to G1, I2-I4 correspond to I1 and K2-K4 correspond to /25
K1), with a homology of 80% or greater being preferred, and a homology of 90% or greater being additionally desirable, and comprises a sequence consisting of at least 10 bases.

PCR primers Y2-Y4 (Y is selected from any of B, D, F, H, J and L, B2 is described in (18), B3 is described in (19), B4 is described in (20), D2 is described in (26), D3 is described in (27), D4 is described in (28), F2 is described in (34), F3 is described in (35), F4 is described in (36), H2 is described in (42), H3 is described in (43), H4 Is described in (44), J2 is described in (50), J3 is described in (51), J4 is described in (52), L2 is described in (58), L3 is described in (59) and L4 is described in (60)) has a homology of 70% or greater with respect to the base sequence of PCR primer Y1 (Y in Y1 and Y2-Y4 denotes a group represented by the same letter of the alphabet; where B2-B4 correspond to B1, D2-D4 correspond to D1, F2-F4 correspond to F1, H2-H4 correspond to H1, J2-J4 correspond to J1 and L2-L4 correspond to L1), with a homology of 80% or greater being preferred, and a homology of 90% or greater being additionally desirable, and comprises a sequence consisting of at least 10 bases.

Any one of the PCR primers X1-X4 (where X is selected from A, C, E, G, I and K) and any one of the PCR primers Y1-Y4 (Y is selected from any of B, D, F, H, J or L) can be used as primers when carrying out PCR or RT-PCR.

As stated above, it is conjectured that six structural genes (mlcA, mlcB, mlcC, mlcD, mlcE and mlcR) are present on the recombinant DNA vector pML48 insertion sequence obtained in the present invention. The cDNA of these six structural genes can be obtained by RT-PCR involving PCR using at any one of PCR primers Y1-Y4 and any one of PCR primers X1-X4 in a reverse transcription reaction. In addition, the various structural genes can be obtained by PCR using ML-236B-producing microorganism genomic DNA along with any one of PCR primers X1-X4 and any one of PCR primers Y1-Y4.

It is appropriate to use any one of the PCR primers Y1-Y4 and any one of the PCR /26
primers X1-X4 described in (1) below as PCR primers for obtaining cDNA that is full length and can be expressed in appropriate host cells.

(1) In obtaining mlcA cDNA, it is desirable to use a combination of any one of PCR primers B1-B4 and any one of PCR primers A1-A4.

In obtaining the mlcB cDNA, it is appropriate to use a combination of any one of PCR primers C1-C4 and any one of PCR primers D1-D4.

In obtaining mlcC cDNA, it is appropriate to use a combination of any one of PCR primers E1-E4 and any one of PCR primers F1-F4.

In obtaining mlcD cDNA, it is appropriate to use any one of PCR primers G1-G4 and any one of PCR primers H1-H4.

In obtaining mlcE cDNA, it is appropriate to use any one of PCR primers I1-I4 and any one of PCR primers J1-J4.

In obtaining mldR cDNA, it is appropriate to use any one of PCR primers K1-K4 and any one of PCR primers L1-L4.

In addition, the requirement of (2) below also applies to primers X1-X4.

(2) PCR primers X1-X4 are designed so that the PCR product obtained using any one of PCR primers X1-X4 and any one of PCR primers Y1-Y4 comprises a translation initiation codon ATG at the original site, and does not have a translation termination codon at a position other than the original position in the translation frame initiated at the translation initiation codon (where Table 4presents the sites of the translation initiation codons for various structural genes hypothesized by the present invention in the base sequence represented by Nucleotide Nos. 1-34203 in Sequence No. 2 of the sequence table and the base sequence represented by Nucleotide Nos. 1-34203 of Sequence No. 1 of the sequence table). /27

The PCR primer X1 has, as its 5'-terminus, the A of the cDNA translation initiation codon ATG or a base farther towards the 5' side.

The PCR primers X2-X4 anneal selectively with a specific region on the base sequence represented by Nucleotide Nos. 1-34203 of Sequence No. 1 of the sequence table or on the base sequence represented by Nucleotide Nos. 1-34203 of Sequence No. 2 of the sequence table (the entire base sequence of Sequence No. 2 of the sequence table is completely complementary with respect to the entire base sequence of Sequence No. 1 of the sequence table).

When PCR primers X2-X4 contain a base sequence farther to the 3' side from the translation initiation codon ATG, the sequence does not comprise a base sequence that will serve as a termination codon (TAA, TAG or TGA) in the translation frame beginning with initiation codon ATG in the base sequence which his present to the 3' side relative to the translation initiation codon ATG. The term "translation frame starting form the initiation codon ATG" refers to a sequence comprising three bases which arises when the base sequence on the 3' side of the

translation initiation codon ATG is divided in units of three bases starting from the translation initiation codon ATG.

When the PCR primers X2-X4 comprise a base or base sequence ("base or base sequence m'" below) corresponding to the A, AT or ATG ("base or base sequence m" below) of the translation initiation codon at this site, if the base or base sequence m is A, then the base or base sequence m' is A, and in addition, the A of the base or base sequence m' is located at the 3' terminus of the PCR primers X2-X4. When the base or base sequence m is AT, then the base or base sequence m' is AT, and in addition, the AT of the base or base sequence m' is situated at the 3'-terminus of the PCR primers X2-X4. When the base or base sequence m is ATG, then the base or base sequence m' is ATG, and, in addition, taking the A of the ATG of the base or base sequence m' as the first and adding in the 3' direction, when a trinucleotide having nucleotide 3n+1 (where n is an integer of 1 or greater) as the 5'-terminus is present in the PCR primers X2-X4, then the base sequence of this trinucleotide is not TAA, TAG or TGA.

Taking the A of the translation initiation codon ATG as the first and adding in the 3'-direction, when the 3'-terminus of PCR primers X2-X4 is nucleotide 3n+1 (where n is an integer /28
of 1 or greater), , then the base sequence of the trinucleotide consisting of the 3n+1 nucleotide and the dinucleotide towards the 3' side thereof is not TAA, TAG or TGA in the RT-PCR product obtained using RNA or mRNA of ML-236B-producing microorganism as template with PCR primers X2-X4 as one of the primers, or PCR product obtained using genomic DNA or cDNA as template.

Taking the A of the translation initiation codon ATG as the first and adding in the 3'-direction, when either one of the 3'-termini of PCR primers X2-X4 is nucleotide 3n+2 (n denotes an integer of 1 or greater), then in the RT-PCR product obtained using RNA or mRNA of ML-236B-producing microorganism as template with PCR primers X2-X4 as one of the primers, or PCR product obtained using genomic DNA or cDNA as template, the base sequence of the trinucleotide consisting of the 3n+2 nucleotide and the two adjacent mononucleotides to the 3' side and the 5' side is not TAA, TAG or TGA.

Taking the A of the translation initiation codon ATG as the first and adding in the 3' direction, when the 3'-terminus of PCR primers X2-X4 is nucleotide 3n+3 (n is an integer of 1 or greater), then the base sequence of the trinucleotide consisting of nucleotides 3n+1 to 3n+3 is not TAA, TAG or TGA.

The above is condition (2).

In addition, condition (3) below applies to PCR primers Y1-Y4.

(3) PCR primers Y1-Y4 are designed so that the cDNA encoded from the N terminus to the C terminus of the peptide encoded by the respective structural genes (mlcA, mlcB, mlcC, mlcD,

mlcE and mlcR) can be amplified by means of PCR using, as primers, any one of PCR primers X1-X4 and any one of PCR primers Y1-Y4.

PCR primer Y1 has no particular restrictions, provided that it is a PCR primer that has a base sequence that is complementary to the base sequence in the vicinity of the cDNA translation /29
termination region, but it is preferable for the primer to have a base sequence having, as its 5'-terminus, a base that is complementary to the base of the 3'-terminus of the translation termination codon or a base that is farther to the 5' side thereof, and it is more preferable for the primer to have a three-base sequence that is complementary with respect to the translation termination codon (Tables 8-10 present the translation termination codons of the various structural genes hypothesized in the present invention, the complementary sequences of the translation termination codons, the C-terminal amino acid residues of the peptides encoded by the various structural genes, the base sequences encoding these amino acid residues and the locations thereof on the base sequences represented by Nucleotide Nos. 1-34203 of Sequence No. 1 of the sequence table and on the base sequences represented by Nucleotide Nos. 1-34203 of Sequence No. 2 of the sequence table).

PCR primers Y2-Y4 selectively anneal with specific regions on the base sequence represented by Nucleotide Nos. 1-34203 of Sequence No. 1 of the sequence table and the base sequence represented by Nucleotide Nos. 1-34203 of Sequence No. 2 of the sequence table.

The above is condition (3).

In addition, with the PCR primers X2-X4 and PCR primers Y2-Y4, it is possible to add appropriate base sequences to the 5'-ends, provided that the conditions of (2) and (3) and the above definitions are satisfied. Examples of these types of base sequences include base sequences that are useful for carrying out subsequent cloning processes on PCR products, and there are no particular restrictions, provided that the primers can be used in PCR. Base sequences that may be cited are restriction enzyme recognition sequences and base sequences containing these restriction enzyme recognition sequences.

In addition, design of PCR primers X1-X4 and PCR primers Y1-Y4 is carried out according to the above description pertaining to the design of PCR primers.

Functional expression of recombinant DNA vectors present in positive clones can be carried out by transforming cells with the recombinant DNA vector, and then measuring the ML-236B production capacity of the transformed cells. Examples of cells used for functional expression are the above ML-236B-producing microorganisms or ML-236B-nonproducing microorganisms. There are no particular restrictions on ML-236B-nonproducing /30
microorganisms, provided that the cells can be transformed with the DNA vector, and examples that may be cited include non-producing modified strains of the above ML-36B-producing

microorganisms. If production of ML-236B is recovered when a modified strain is transformed, then it can be surmised that the recombinant DNA vector has the desired function.

The transformation method used for functional expression is selected appropriately in connection with the host cells. Transformation of *Penicillium citrinum*, which is a preferred ML-236B-producing microorganism, can be carried out by preparing protoplasts from *Penicillium citrinum* spores, and then introducing the recombinant DNA vector into the protoplasts (Nara, F. et al., Curr. Genet. 23, 28 (1993)).

Preparation of the protoplasts is carried out by the method described below.

Cells from a *Penicillium citrinum* slant culture are used in order to inoculate PGA agar medium plates, which are maintained at a temperature of 22-28°C for 10-14 days. The spores are then recovered and 1 x 10^7 to 1 x 10^9 spores are used in order to inoculate 50-100 mL of YPL-20 medium (composition: 0.1 (w/v) yeast extract (Difco), 0.5% (w/v) polypeptone (Nippon Seiyaku), 20% (w/v) lactose, pH 5.0), whereupon a temperature of 22-28°C is maintained for 18 h to 2 days. The generates pores are then recovered from said culture, and are treated with cell wall digestion enzyme to obtain protoplasts. There are no particular restrictions on the cell wall digesting enzyme, provided that the enzyme breaks down *Penicillium citrinum* cell wall and does not adversely act on the microorganisms. Examples that may be cited include zymolyase and chitinase.

Culturing of the transformed ML-236B-producing microorganisms can be carried out under conditions appropriate for the host cells, but with transformants of *Penicillium citrinum*, which is a preferred ML-236B-producing microorganism, the cell walls are regenerated beforehand by culturing the transformed microorganisms protoplasts under appropriate conditions prior to production of ML-236B.

Regeneration of the cell walls can be carried out by sandwiching a VGS middle-layer agar medium containing *Penicillium citrinum* protoplasts (composition: Vogel minimal medium, 2% (w/v) glucose, 1 M glucitol, 2% (w/v) agar) between a VGS bottom layer agar medium (composition: Vogel minimal medium, 2% (w/v) glucose, 1 M glucitol, 2.7% (w/v) agar) /31
and VGS top layer agar medium (composition: Vogel minimal medium, 2% (w/v) glucose, 1 M glucitol, 1.5% (w/v) agar) and maintaining a temperature of 22-28°C for 7-15 days. The resulting strain is then cultured for a generation on PGA medium while maintaining a temperature of 22-28°C. A slant produced from PGA medium is then inoculated with a platinum loop of this strain, and is maintained at a temperature of 22-28°C for 10-14 days, and stored at 0-4°C.

Transformant from a slant produced by culturing the *Penicillium citrinum* transformant with the regenerated cell walls described above is then used in order to inoculate MBG3-8 medium, and by maintaining a temperature of 22-28°C while shaking for 7-12 days, it is possible

to efficiently generate ML-236B. With *Penicillium citrinum* host cells, it is possible to produce ML-236B by means of exactly the same liquid culturing process.

Purification of ML-236B from culture of ML-236B-producing microorganisms involves a combination various techniques used in common natural product purification. There are no particular restrictions on these various techniques, and examples that may be cited include centrifugal separation, solid/liquid separation by means of filtration, alkali or acid treatment, extraction using organic solvents, solvent exchange, adsorption, fractionation and various type of chromatographic processes, and crystallization. ML-236B has two interconvertible forms. In addition, the hydroxy acid form produces a stable salt. By utilizing these physiochemical characteristics, it is possible to obtain ML-236B hydroxy acid ("free hydroxy acid" below), ML-236B hydroxy acid salt ("hydroxy acid salt" below) or ML-236B lactone ("lactone" below).

The compound is subjected to ring opening by alkali hydrolysis of the culture under heat or at common temperatures, thereby converting the compound to hydroxy acid salt. After acidification of the reaction solution and filtration, the filtrate is then extracted with an organic solvent that is not miscible with water, thereby obtaining the target compound as free hydroxy acid. There are no particular restrictions on organic solvents that are not miscible with water, and examples that may be cited include hexane, heptane and other aliphatic hydrocarbons, benzene, toluene and other aromatic hydrocarbons, methylene chloride, chloroform and other halogenated hydrocarbons, diethyl ether and other ethers, ethyl formate, ethyl acetate and other esters, as well as mixed solvents consisting of two or more types of these solvents. /32

In addition, the free hydroxy acid can be switched into an aqueous solvent of alkali metal salt such as sodium hydroxide, thereby obtaining the target compound as a hydroxy acid salt.

This free hydroxy acid can also be subjected to ring opening by heating in organic solvent and dehydration or other method, thereby obtaining the target compound as lactone.

The free hydroxy acid, hydroxy acid salt or lactone obtained in this manner can be purified and isolated by column chromatography or other method. There are no particular restrictions on column chromatography matrices, but examples that may be cited include Sephadex LH-20 (Pharmacia), Diaion HP-20 (Sanyo Chemical), silica gel, and reverse-phase system matrices, with C^{18}-based matrices being preferred.

There are no particular restrictions on the method for quantifying the ML-236B, provided that the method is commonly used in the quantification of organic compounds. Examples that may be cited include reverse-phase high-performance liquid chromatography ("reverse phase HPLC" below). Quantification by means of reverse-phase HPLC can be carried out by alkali hydrolysis of ML-236B-producing microorganism culture, and then subjecting the soluble fraction to reverse-phase HPLC using a C^{18} column. The ultraviolet absorption may be measured, and this absorption may be quantified. There are no particular restrictions on the C^{18}

column, provided that the C^{18} column is commonly used in reverse-phase HPLC, and an example that may be cited is the SSC-ODS-262 column (diameter 6 mm, length 100 mm; manufactured by Senshu Chemical). There are no particular restrictions on the mobile phase, provided that it is a solvent that is commonly used in reverse-phase HPLC, and an example that may be cited is 75% (v/v) methanol-0.1% (v/v) triethylamine-0.1% (v/v) triethylamine-0.1% (v/v) acetic acid. When 75% (v/v) methanol-0.1% (v/v) triethylamine-0.1% (v/v) triethylamine-0.1% (v/v) acetic acid is used as mobile phase at a flow rate of 2 mL/min, the compound will elute at 4.0 min after applying the ML-236B to the SSC-ODS-262 column at room temperature. Detection of ML-236B can be carried out using an HPLC UV detector, and the absorption wavelength of the UV detector is 220-280 nm, preferably 220-260 nm, and more preferably 236 nm. /33

The desired recombinant DNA vector having genes that are confirmed to be functionally expressed is useful in the improvement of ML-236B production.

In this specification, adenine is denoted by "A", guanine is denoted by "G", thymine is denoted by "T" and cytosine is denoted by "C". The base sequences represented by the various Sequence Nos. of the sequence tables are described in "Guidelines for Drafting Specifications Containing Base Sequences and Amino Acid Sequences" (Japanese Patent Office, June 1998).

Brief description of the figures

Figure 1 is a construction diagram of the DNA vector pSAKcos1 which can be introduced into *E. coli* and yeast, and which allows insertion of long DNA sequences.

Figure 2 is a structural gene analysis of the pML48 insertion sequence.

Figure 3 is a Northern blot hybridization of the pML48 insertion sequence.

Best embodiment of the invention

The resent invention is described in additional detail below by providing application examples and test examples, but the present invention is not restricted to these examples.

Application example 1. Production of the pSAKcos1 vector

1) Plasmid pSAK333 (Japanese Kokai Patent Application No. Hei 3[1991]-262486) containing the hygromycin B phosphotransferase gene derived from *E. coli* was digested with the restriction enzyme BamHI (Takara Shuzo) and the terminals were blunted using T4 DNA polymerase (Takara Shuzo).

2) DNA Ligation Kit Ver. 2 (Takara Shuzo) was used in order to bring about autocyclization of the above DNA fragment, and the material was then used in order to transform E col. strain JM109 competent cells (Takara Shuzo). Strains harboring plasmids with deleted BamHI sites were selected from the transformed *E. coli* cells, and the plasmid contained in this

strain was named pSAK360. /34

3) After digesting pSAK360 with restriction enzyme PvuII, the 5'-terminals were dephosphorylated by alkali phosphatase treatment. The SalI-ScaI fragment (approximately 3 kb) having a (cos) site was obtained from cosmid vector pWE15 (Stratagene) and after blunting the terminals with T4 DNA polymerase, the fragment was ligated at the PvuII site of pSAK360 and was used in order to transform strain JM109. Strains harboring the plasmid in which the SalI-ScaI fragment from the above transformed *E. coli* cells (approximately 3 kb) had been inserted at the PvuII site were selected and the plasmid contained in the strain was named "pSAKcos1". pSAKcos1 has one each of the restriction enzyme recognition sites BamHI, EcoRI and NotI derived from pWE15. In addition, pSAKcos1 has an ampicillin resistance gene and hygromycin resistance gene as selection markers. In the following application examples, when *E. coli* is used as host, selection of transformants transformed with pSAKcos1 or pSAKcos1 containing exogenous inserted DNA was carried out by the addition of 40 μg/mL of ampicillin to the medium. When *Penicillium citrinum* SANK13380 was used as host, selection of transformants transformed with pSAKcol or pSAKcos1 containing exogenous inserted DNA was carried out by adding 200 μg/mL of hygromycin B to the medium.

The procedure for constructing pSAKcos1 is presented in Figure 1.

Application example 2. Preparation of genomic DNA from *Penicillium citrinum* stain SANK13380

1) Culturing of *Penicillium citrinum* strain SANK13380

Culturing of *Penicillium citrinum* strain SANK13380 inoculum was carried out on a slant using PGA agar medium. Specifically, a platinum loop was used in inoculation using *Penicillium citrinum* strain SANK13380, and a temperature of 26°C was maintained for 14 days. The slant was stored at 4°C.

The culture was then subjected to aerated liquid culturing. A 500-mL Erlenmeyer flask /35
containing 50 mL of MGB3-8 medium was inoculated with microorganism cells from a 5 mm^2 slant, and culturing was carried out while shaking for 5 days under conditions of 26°C and 210 rpm.

2) Preparation of genomic DNA from *Penicillium citrinum* strain SANK13380 culture

1) The culture product was centrifuged for 10 min under conditions of room temperature and 1000 xg, and the microorganism cells were collected. 3 g, wet weight, of the microorganism cells were then chilled on dry ice and ground with a mortar and pestle to produce a powder. The ground cells were then introduced into a centrifuge tube containing 20 mL of 62.5 mM EDTA·2Na (Wako Pure Chemical)-5% (w/v) SDS-50 mM Tris (Wako Pure

chemical)-hydrochloride acid (Wako Pure chemical) buffer (pH 8.0), and after mixing gently, the solution was left for 1 h at 0°C. 10 mL of phenol neutralized with 10 mM Tris-HCl-0.1 mM EDTA·2Na (pH 8.0; "TE" below) was then added, and the solution was stirred gently for 1 h at 50°C. After centrifuging for 10 min under conditions of room temperature and 10,000 xg, 15 mL of supernatant (aqueous phase) was transferred into a separate centrifuge tube, and a solution of 0.5 volumes of TE saturated phenol and 0.5 volumes of chloroform was added. After stirring gently for 2 min, the solution was centrifuged for 10 min under conditions of room temperature and 10,000 xg ("phenol-chloroform extraction" below). 10 mL of 8 M ammonium acetate (pH 7.5) and 25 mL of 2-propanol (Wako Pure Chemical) were then added to 10 mL of supernatant, and after chilling for 15 min at -80°C, the material was centrifuged for 10 min under conditions of 4°C and 10,000 xg. The sediment was then dissolved in 5 mL of TE, 20 μL of 10 mg/mL ribonuclease A (Sigma) and 250 U of ribonuclease T1 (GIBCO) were added, and the material was maintained at 37°C for 20 min. 20 mL of 2-propanol were then added, and after mixing gently, the filamentous genomic DNA was taken up within a Pasteur pipette and was then dissolved in 1 mL of TE. 0.1 volume of 3 M sodium acetate (pH 6.5) and 2.5 volumes of ethanol were then added to this DNA, and after chilling for 15 min at -80°C, the material was centrifuged for 5 min under conditions of 4°C and 10,000 xg ("ethanol precipitation" below). The resulting sediment was dissolved in 200 μL of TE to produce a genomic DNA fraction.

<u>Application example 3. Production of *Penicillium citrinum* strain SANK13380 genomic DNA</u> /36
<u>library</u>

1) Preparation of genomic DNA fragments

0.25 U of Sau3AI (Takara Shuzo) was added to 100 μL of a solution containing the genomic *Penicillium citrinum* strain SANK13380 DNA (50 μg) obtained in Application example 2, and after 10 sec, 30 sec, 60 sec, 90 sec and 120 sec, 20 μL amounts were sampled, and 0.5 M EDTA (pH 8.0) was added in the amount of 20 μL to each sample to stop the restriction enzyme reaction. The resulting partially digested DNA fragments were then separated by agarose gel electrophoresis, and the agarose gel containing DNA fragments having sizes of 30 kb or greater was recovered.

The recovered gel was crushed into small pieces and was introduced into an Ultrafree-C3 centrifuge filtration unit (Japan Millipore). After chilling for 15 min at -80°C to freeze the gel, the gel was then melted by maintaining it for 10 min at 37°C. The material was then centrifuged for 5 min at 5000 xg to obtain a DNA extract. The DNA extract was then subjected to phenol-chloroform extraction and ethanol precipitation, and the resulting precipitate was dissolved in a small quantity of TE.

2) DNA vector pSAKcos1 pretreatment

After digesting pSAKcos1 with restriction enzyme BAMHI (Takara Shuzo), an alkali phosphatase (Takara Shuzo) reaction was carried out for 30 min at 65°C. The solution obtained upon completion of the reaction was subjected to phenol-chloroform extraction and ethanol precipitation, and the resulting precipitate was dissolved in a small quantity of TE.

3) Ligation and *in vitro* packaging

Genomic DNA fragment from (1) above (2 μg) and the above completed pSAKcos1 (1 μg) from (2) above (1 μg) were mixed, and DNA Ligation Kit Ver. 2 (Takara Shuzo) was used in order to carry out at a 16-h ligation reaction at 16°C. The solution obtained upon completion of the reaction was then subjected to phenol-chloroform extraction and ethanol precipitation, and the resulting precipitate was dissolved in 5 μL of TE. Ligation product solution was subjected to *in vitro* packaging using Gigapak II Gold (Stratagene), and transformed *E. coli* containing /37
recombinant DNA vector was obtained. 3 mL of LB medium was then introduced into a plate on which colonies of the transformed *E. coli* had been formed, and the colonies on the plate were recovered using a cell separator (recovered solution (1)). In addition, the plate was washed with 3 mL of LB medium, and the solution was recovered (recovered solution (2)). Glycerin was added to a mixture of the recovered solutions (1) and (2) to produce a final concentration of 18%. The resulting solution was referred to as "*E. coli* solution," and was stored at -80°C as strain SANK13380 genomic DNA library.

<u>Application example 4. Amplification PKS gene by means of PCR using *Penicillium citrinum* strain SANK13380 genomic DNA as template</u>

1) PCR primer design and synthesis

Mixed primers represented by Sequence Nos. 3 and 4 of the sequence table were designed based on the amino acid sequence of the PKS gene of *Aspergillus flavus* (Brown, D.W., et al., Proc. Natl. Acad. Sci. USA 93, 1418 (1996)).

Sequence No. 3 of the sequence table: GAYACNGCNTGYASTTC

Sequence No. 4 of the sequence table: TCNCCNKNRCWGTGNCC

In the base sequence represented by Sequence Nos. 3 and 4 of the sequence table, N denotes an inosine base (hypoxanthine), Y denotes T or C, S denotes G or S, K denotes G or T, R denotes G or A and W denotes A or T.

2) Amplification of DNA fragments by PCR

50 μL of reaction solution containing the PCR primers described in (2) above (100 pmol each), genomic DNA from *Penicillium citrinum* strain SANK13380 obtained in Application

Example 2 (500 ng), 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 50 mM potassium chloride, 2 mM magnesium chloride and 1.25 U Ex. Taq DNA polymerase (Takara Shuzo) was subjected to a reaction cycle comprising a series of three continuous steps of 1 min at 94°C, 2 min at 58°C and 3 min at 70°C. This cycle was repeated 30 times, thereby amplifying /38
the DNA fragment. PCR was carried out using a TP3000 TaKaRa PCR Thermal Cycler (Takara Shuzo).

The amplified DNA fragments were subjected to agarose gel electrophoresis, and agarose gel containing DNA fragments having sizes of about 1.0-2.0 kb was recovered. DNA was recovered from the gel, and after phenol-chloroform extraction and ethanol precipitation, the resulting precipitate was dissolved in a small quantity of TE.

3) Ligation and transformation

The DNA fragments obtained in (2) were used along with TA cloning system pCR2.1 (Invitrogen), and the fragments were ligated with plasmid pCR2.1 provided in the kit to obtain transformed strains.

A number of the resulting clones were selected, and were cultured by the method described by Maniatis et al. (Maniatis, T. et al., Molecular Cloning, A laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989)). Specifically, 24 mL test tubes containing 2 mL of LB medium were inoculated with respective colonies and the material was cultured for 18 h at 37°C while shaking.

Preparation of recombinant DNA vectors from the culture was carried out by the alkali method (Maniatis, T. et al., Molecular Cloning, A laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989)). Specifically, 1.5 mL of culture solution was centrifuged for 2 min under condition of room temperature and 10,000 xg, and the cells were recovered form the sediment. 100 μL of 50 mM glucose-25 mM Tris-HCl-10 mM EDTA (pH 8.0) was added to the cells and the cells were suspended. Subsequently, 200 μL of 0.2 N sodium hydroxide-1% (w/v) SDS was added and stirred gently to lyse the cells. 150 μL of 3 M potassium acetate-11.5% (w/v) glacial acetic acid was then added in order to denature the protein, and the material was then centrifuged for 10 min under conditions of room temperature and 10,000 xg, whereupon the supernatant was recovered. The supernatant was subjected to phenol-chloroform extraction and ethanol precipitation, and the resulting precipitate was then dissolved in 50 μL of TE containing 40 μg/mL of ribonuclease A (Sigma).

The recombinant DNA vectors were then digested with restriction enzyme and /39
electrophorosed, whereupon the insertion base sequence in the recombinant DNA vectors of different electrophoresis patterns were analyzed using a DNA sequencer (model 377 Perkin Elmer Japan).

As a result, it was confirmed that strains were present that carried the recombinant DNA vector containing the PKS gene fragment.

Application Example 5. Genomic Southern blot hybridization of *Penicillium citrinum* strain SANK13380.

1) Electrophoresis and transfer onto membranes

Genomic DNA (10 μg) from *Penicillium citrinum* strain SANK13380 obtained in Application Example 2 was digested using restriction enzymes EcoRI, SalI, HindIII or SacI (all manufactured by Takara Shuzo), and the material was then subjected to agarose gel electrophoresis. Agarose L03 "Takara" (Takara Shuzo) was used in preparing the agarose gel. After electrophoresis, the gel was immersed in 0.25 N hydrochloric acid (Wako Pure Chemical) and was shaking gently for 10 min at room temperature. The gel was then transferred into 0.4 N sodium hydroxide (Wako Pure Chemical) and was shaken gently for 30 min at room temperature. The DNA in the gel was then transferred onto nylon membrane Hybond™-N+ (Amersham) using the alkali transfer method of Maniatis et al. (Maniatis, T. et al., Molecular Cloning, A laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989)), and was fixed thereupon. The membrane was then washed with 2x SSC (1x SSC composition: 150 mM NaCl, 15 mM trisodium citrate) and was air dried.

2) Hybridization and signal detection

Hybridization with respect to the membrane obtained in (1) was then carried out using the PKS gene fragments obtained in Application Example 4 as probes.

Probes were used which were produced by labeling PKS gene DNA fragments obtained in Application Example 4 (1 μg) using the DIG DNA Labeling Kit (Boehringer-Mannheim). The material was used after boiling for 10 min and allowing to cool immediately prior to use.

The membrane described in (1) was immersed in hybridization solution (DIG Easyhyb; /40
Boehringer-Mannheim), and prehybridization was allowed to occur for 2 h at 42°C while agitating at 20 rpm. The above labeled probes were then added to the hybridization solution, and hybridization was allowed to occur for 18 h at 42°C while agitating at 20 rpm using a Multishaker Oven HB (TAITEC). They hybridized membrane was then washed three times for 20 min at room temperature using 2x SSC, and was washed twice at 55°C for 30 min using 0.1x SSC.

The washed membrane was then treated with DIG Luminescent Detection Kit for Nucleic Acids (Boehringer Mannheim) and exposure was carried out on x-ray film (Lumifilm; Boehringer-Mannheim). Development was carried out using an FPM800A Fuji Medical Film Processor (Fuji Film).

As a result, it was confirmed that the PKS gene fragment obtained in Application Example 4 was present on the *Penicillium citrinum* genome.

<u>Application Example 6. Screening of *Penicillium citrinum* strain SANK13380 genomic DNA using the PKS gene fragment as probe</u>

Cloning of genomic DNA containing the PKS gene was carried out by the colony hybridization method.

1) Preparation of membranes

E. coli bacterial solution (from Application Example 3) which had been stored as *Penicillium citrinum* strain SANK13380 genomic DNA library was diluted so that 5000-10,000 colonies were generated per plate of LB agar medium, and after maintaining the plates at a temperature of 26°C for 18 h, the plates were cooled to 4°C over a period of 1 h. Hybond™-N+ (Amersham) was then placed on a plate, and was allowed to be in contact therewith for 1 min. The membrane with the attached colonies was then carefully peeled from the plate and was immersed for 7 min in 200 mL of 1.5 M sodium chloride-0.5 N sodium hydroxide, with the /41
colony contact surface upwards. The membrane was then immersed twice, three minutes each time, in 200 mL of 1.5 M sodium chloride-0.5 M Tris-HCl-1 mM EDTA (pH 7.5), followed by washing with 400 mL of 2x SSC. The washed membrane was then allowed to air dry for 30 min.

2) Hybridization

The probes were produced by using a DIG DNA Labeling Kit (Boehringer-Mannheim) to label PKS gene fragment DNA (91 μg) obtained in Application Example 4 and immediately prior to use, were boiled for 10 min and allowed to cool.

The membrane from (1) was immersed in hybridization solution (DIG Easyhyb; Boehringer-Mannheim), and prehybridization was allowed to occur for 2 h at 42°C while agitating at 20 rpm. The above labeled probes were then added to the hybridization solution, and hybridization was allowed to occur for 18 h at 42°C while agitating at 20 rpm using a Multishaker Oven HB (TAITEC). They hybridized membrane was then washed three times for 20 min at room temperature using 2x SSC, and was washed twice at 68°C for 30 min using 0.1x SSC.

The washed membrane was then treated with DIG Luminescent Detection Kit for Nucleic Acids (Boehringer Mannheim) and exposure was carried out on x-ray film (Lumifilm; Boehringer-Mannheim). Development was carried out using Fuji medical film processor FPM800A (Fuji Film).

The procedures described in (1) and (2) are referred to as screening.

The region around the colonies for which a positive signal was detected in the first screening were collected, and after suspending in LB medium, appropriate dilution was carried out and the material was spread onto plates and cultured. Secondary screening was then carried out in the same manner in order to purify the positive clones.

The positive clones obtained in this application example, specifically, transformed *E. coli* strain pML48 SANK71199, was internationally deposited on July 7, 1999 under Accession No. FERM BP-6780 at the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Japan Ministry of International Trade and Industry (1-3, Tsukuba-shi 1-chome, Ibaraki-ken, Japan). /42

Application Example 7. Analysis of the recombinant DNA vector pML48 insertion sequence (1)

Culturing of the *E. coli* strain pML48 SANK71199 obtained in Application Example 6 and preparation of recombinant DNA vector from the culture were carried out according to the method described in Application Example 4.

The resulting recombinant DNA vector was named pML48. The pML48 insertion sequence was digested with various restriction enzymes and subcloning was carried out by introducing it into pUC119 (Takara Shuzo). The resulting subclones were used as probes and Southern blot hybridization was carried out according to the method described in Application Example 5. Specifically, the various restriction enzyme digestion products of pML48 were subjected to electrophoresis and hybridization was carried out with respect to membranes produced by transferring the DNA onto membranes.

As a result, a restriction enzyme map of the pML48 insertion sequence was produced.

In addition, the base sequences of the insertion sequences of the above various subclones were identified using a model 377 DNA sequencer (Perkin Elmer Japan), and the entire base sequence of pML48 was thereby determined.

The insertion sequence of pML48 was 34203 bases.

The base sequence of the pML48 insertion sequence is described in Sequence Nos. 1 and 2 of the sequence table. The base sequences presented in Sequence Nos. 1 and 2 of the sequence table are completely complementary to each other.

Regarding the presence of structural genes on the insertion sequence, analysis was carried out using the GRAIL gene detection program (ApoCom GRAIL Toolkit; APOCOM) and the BLAST homology detection program (Gaped-BLAST (BLAST2); included in Wisconsin GCG Package Ver. 10.0) .

As a result, it was conjectured that six different structural genes were present in the pML48 insertion base sequence, and these genes were named mlcA, mlcB, mlcC, mlcD, mlcE and mlcR. In addition, it was conjectured that mlcA, mlcB, mlcC, and mlcR had coding regions /43

in the base sequence of Sequence No. 2 of the sequence table, and that mlcC and mlcD had coding regions in the base sequence represented by Sequence No. 1 of the sequence table. In addition, the relative positions and sizes of each of the presumed structural genes in the insertion sequences were conjectured.

The results of this application example are presented in Figure 2.

Application Example 8. Analysis of recombinant DNA vector pML48 insertion sequence (2)

Expression analysis of the structural genes suggested in Application Example 7 as well as analysis of the 5'-terminal and 3'-terminal regions were carried out by means of Southern blot hybridization and RACE.

1) Preparation of *Penicillium citrinum* SANK13380 total RNA

A 5 mm^2 section of cells was collected from a slant on which *Penicillium citrinum* strain SANK13380 had been cultured (Application Example 2), and the cells were used in order to inoculate 10 mL of MGB3-8 medium contained in a 100-mL Erlenmeyer flask, whereupon culturing was carried out at 26°C for 3 days while shaking.

Preparation of total RNA from the culture was carried out using the RNeasy Plant Mini Kit (Qiagen) by means of the guanidine isothiocyanate method. Specifically, the culture was centrifuged for 10 min under conditions of room temperature and 5000 xg to recover the cells, and after freezing 2 g, wet weight, of cells on liquid nitrogen and grinding into a powder using a mortar and pestle, the ground material was suspended in 4 mL of cell solution buffer (contained in the kit) containing guanidine-isothiocyanate. The suspension was then aliquotted in 450 μL amounts into ten QIAshredder spin columns provided in the kit, and after centrifuging for 10 min at 1000 xg and room temperature, the eluates were recovered. 225 μL amounts of ethanol were added to each eluate, and this was then added to RNA Minispin columns provided in the kit. After washing the columns with wash buffer contained in the kit, 50 μL amounts of ribonuclease-free purified water were used in order to elute the adsorbed material. The eluate was used as the total RNA fraction.

2) Northern blot hybridization

1 μL of 10x MOPS (composition 200 mM 3-morpholinopropanesulfonic acid, 50 mM /44
sodium acetate, 10 mM EDTA·2Na, pH 7.0; used after sterilizing in an autoclave for 20 min at 121°C; Dojindo), 1.75 μL of formaldehyde and 5 μL of formamide were added to 2.25 μL of an aqueous solution containing 20 μg of *Penicillium citrinum* SANK13380 total RNA and mixed to produced an RNA sample. The RNA sample was then maintained for 10 min at 65°C, before chilling rapidly on ice water and subjecting the material to agarose gel electrophoresis. The

electrophoresis gel was produced by mixing 10 mL of 10x MOPS and 1 g of Agarose L03 "Takara" (Takara Shuzo)in 72 mL of water treated with pyrocarbonic acid diethyl ester (Sigma), and, after heating to dissolve the agarose, cooling the material and adding 18 mL of formaldehyde. The sample buffer was 1x MOPS (10x MOPS diluted 10x with water). The RNA in the gel was then transferred onto Hybond^{TM}-N+ (Amersham) in 10x SSC.

DNA fragments (a, b, c, d and e) obtained by digesting the pML48 insertion sequence with restriction enzymes 1 and 2 presented in Table 1 below were used.

Table 1. Northern blot hybridization probes

① プローブ	② 制限酵素1	③ 制限酵素認識部位のヌクレオチド番号*	④ 制限酵素2	⑤ 制限酵素認識部位のヌクレオチド番号*
a	EcoRI	6319～6324	EcoRI	15799～15804
b	BamHI	16793～16798	PstI	18164～18169
c	KpnI	26025～26030	BamHI	27413～27418
d	SalI	28691～28696	SalI	29551～29556
e	HindIII	33050～33055	SacI	34039～34044

*各ヌクレオチド番号は、配列表の配列番号1に基く。

Key:
1 Probe
2 Restriction enzyme 1
3 Nucleotide Nos. of the restriction enzyme recognition sites
4 Restriction enzyme 2
5 Nucleotide Nos. of the restriction enzyme recognition sites

*The number of each nucleotide is based on Sequence No. 1 of the sequence tables.

Probe labeling, hybridization and signal detection was carried out according to Southern hybridization of Application Example 5.

The results of this application example are described in Figure 3. /45

Each signal indicates the existence of transcription products that are homologous with the base sequence of each probe.

In this application example, it was confirmed that mlcB, mlcD, mlcE and mlcR among the six structural genes hypothesized to be present on the pML48 insertion sequence were transcribed in strain SANK13380, which suggested that mlcA and mlcC were also transcribed in these cells.

The position of each signal does not denote the corresponding size of the transcript.

3) Determination of 5'-terminal sequence by 5'-RACE

cDNA containing the 5'-terminal region of each structural gene was obtained using the 5'-RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (GIBCO).

Two types of antisense oligo-DNA strands were produced which were coding regions designed based on base sequences thought to be located near the 5'-terminus of the genes within the various structural genes on the insertion sequence of pML48 hypothesized based on the results of Application Example 7 and (2) of this application example.

Table 2 shows the base sequences of the antisense oligo-DNA sequences (1) that were designed based on base sequences located on the 3' sides of the structural genes, and Table 3 shows the base sequences of antisense oligo-DNA sequences (2) designed based on base sequences to the 5' side thereof.

Table 2. Oligo-DNA sequences (1) produced using t'-terminus sequence analysis by means of 5'-RACE.

1 遺伝子	2 配列表の配列番号：塩基配列
mlcA	配列番号　5 ：gcatgttcaatttgctctc
mlcB	配列番号　6 ：ctggatcagacttttctgc
mlcC	3 配列番号　7 ：gtcgcagtagcatgggcc
mlcD	配列番号　8 ：gtcagagtgatgctcttctc
mlcE	配列番号　9 ：gttgagaggattgtgagggc
mlcR	配列番号１０ ：ttgcttgtgttggattgtc

Key: 1 Gene
2 Sequence no. from the sequence table; base sequence
3 Sequence no. 5

Table 3. Oligo-DNA sequences (2) produced using 5'-terminus sequence analysis by means of 5'-RACE. /46

1 遺伝子	2 配列表の配列番号：塩基配列
mlcA	3 配列番号１１：catggtactctcgccccgttc
mlcB	配列番号１２：ctccccagtacgtaagctc
mlcC	配列番号１３：ccataatgagtgtgactgttc
mlcD	配列番号１４：gaacatctgcatccccgtc
mlcE	配列番号１５：ggaaggcaaagaaagtgtac
mlcR	配列番号１６：agattcattgctgttggcatc

Key: 1 Gene
2 Sequence no. from the sequence table; base sequence
3 Sequence no. 5

The oligo-DNA (1) used as primer and a cDNA primary chain were synthesized by reverse transcription using total RNA from *Penicillium citrinum* strain SANK13380 as template. Specifically, 24 μL of reaction solution containing 1 μg of total RNA, 2.5 pmol of oligo-DNA (1) and 1 μL of Super Script ™ II Reverse Transcriptase (contained in kit) was maintained at 16°C for 1 h, the product was added to a Glassmax spin cartridge contained in the kit, and cDNA primary strand was purified.

A poly-C chain was added to the 3'-terminus of the cDNA primary strand by means of the terminal deoxyribonucleotidyl transferase contained in the kit.

50 μL of reaction solution containing cDNA primary strand with the poly-C chain added to the 3'-terminus, 40 pmol of oligo-DNA (2) and 40 pmol of Abridged Anchor Primer (included in the kit) was maintained at 94°C for 2 min, and a reaction was then carried out 35 times which comprised 30 sec at 94°C, 30 sec at 55°C and 2 min at 72°C. The reaction was then maintained at 72°C for 5 min and at 4°C for 18 h. The resulting product was subjected to agarose gel electrophoresis, whereupon the DNA was recovered from the gel and the product was purified by phenol-chloroform extraction and ethanol precipitation. The product was then cloned using pCR2.1 according to the method described in Application Example 4.

The above process is referred to as 5'-RACE /47

The base sequence of the cDNA fragment including the 5'-terminus was then determined, and the location of the transcription starting point and the translation initiation codon were hypothesized.

Table 4 presents the sequence table Sequence Nos. for the base sequences of the 5'-terminal cDNA fragments corresponding to the structural genes obtained by 5'-RACE. In addition, Table 5 presents the transcription initiation points, the Sequence Nos. in which the translation initiation points are present, the locations of the transcription initiation points and the locations of the translation starting points.

Table 4. Sequence table Sequence Nos. for the 5'-terminus cDNA fragment base sequences

1 遺伝子	2 配列表の配列番号
mlcA	配列番号１７
mlcB	配列番号１８
mlcC	配列番号１９
mlcD	配列番号２０
mlcE	配列番号２１
mlcR	配列番号２２

Key: 1 Gene
2 Sequence table Sequence No.
3 Sequence No. 17

Table 5. Transcription initiation point and position of the translation initiation codon for each gene.

1 遺伝子番号	2 翻訳開始コドンの存在する配列番号＊	3 配列番号１又は２におけるヌクレオチド 4 転写開始点	5 翻訳開始コドン
mlcA	配列番号２	２２９１３	２３０４５～２３０４７
mlcB	配列番号２	１１６８９	１１７４８～１１７５０
mlcC	配列番号１	１１６４１	１１７９６～１１７９８
mlcD	配列番号１	２４０６６	２４３２１～２４３２３
mlcE	配列番号２	３３９９	３５４５～３５４７
mlcR	配列番号２	３６５	４００～４０２

Key:
1 Gene No.
2 Sequence No. containing the translation initiation codon
3 Nucleotides in Sequence No. 1 and 2
4 Transcription starting point
5 Translation initiation codon
6 Sequence No.

* The base sequences presented in Sequence Nos. 1 and 2 of the sequence table are completely complementary with respect to each other. /48

4) Determination of 3'-terminal sequences by 3'-RACE

cDNA containing the 3'-terminus region of each gene was obtained by using the Ready to Go: T-Primed First-Strand kit (Pharmacia).

One type of sense oligo-DNA (3) was produced which was thought to be a coding region present near the 3'-terminus for each structural gene on the pML48 insertion base sequence hypothesized based on Application Example 7 and (2) of this application example.

Table 6 presents the base sequences of the oligo-DNA strands (3) produced for each structural gene.

Table 6. Oligo-DNA strands (3) used in 3'-terminal sequence analysis by means of 3'-RACE

1 遺伝子	2 配列表の配列番号：塩基配列
mlcA	配列番号２３：atcataccatcttcaacaac
mlcB	配列番号２４：gctagaataggttacaagcc
mlcC	配列番号２５：acattgccaggcacccagac
mlcD	配列番号２６：caacgcccaagctgccaatc
mlcE	配列番号２７：gtcttttcctactatctacc
mlcR	配列番号２８：ctttcccagctgctactatc

Key: 1 Gene
2 Sequence Nos. in the sequence table: Base sequence
3 Sequence No. 23

cDNA primary strands were synthesized by reverse transcription reactions using oligo-DNA (3) as primers and using total RNA from *Penicillium citrinum* strain SANK 13380 (1 μg).

100 μL of reaction solution containing cDNA primary strand, 40 pmol of oligo-DNA (3) and NotI-d(T) 18 primers (contained in the kit) were maintained at 94°C for 2 min, and a reaction was repeated 35 times with 1 cycle comprising 30 sec at 94°C, 30 sec at 55°C and 2 min at 72°C. The reaction was then maintained a 72°C for 5 min and for 18 h at 4°C. The resulting product was subjected to agarose gel electrophoresis, the DNA was recovered form the gel, and /49
the product was purified by phenol-chloroform extraction and ethanol precipitation. The product was then cloned using pCR2.1 based on the method of Application Example 4.

The above procedure is referred to as 3'-RACE

The base sequence of the 3'-side fragment of the resulting cDNA was determined and the position of the translation termination codon was hypothesized.

Table 7 presents the sequence table Sequence Nos. that indicate the base sequences of the 3'-terminal cDNA fragments corresponding to each structural gene obtained by 3'-RACE. In addition, Table 8 presents the translation termination codons and the positions of said codons for the various structural genes based on Sequence Nos. 1 and 2.

Table 7. Sequence table Sequence Nos. indicating the base sequences of the 3'-terminal cDNA fragments

1 遺伝子	2 配列表の配列番号
mlcA	3 配列番号２９
mlcB	配列番号３０
mlcC	配列番号３１
mlcD	配列番号３２
mlcE	配列番号３３
mlcR	配列番号３４

Key: 1 Gene
2 Sequence table Sequence Nos.
3 Sequence no. 29

Table 8. Translation termination codon and position thereof for each structural gene

1 遺伝子	2 翻訳終止コドン	3 翻訳終止コドンの存在する配列番号＊	4 配列番号１又は２における翻訳終止コドンのヌクレオチド番号
mlcA	tag	5 配列番号２	３２７２３～３２７２５
mlcB	taa	配列番号２	１９８４０～１９８４２
mlcC	taa	配列番号１	１３４７９～１３４８１
mlcD	tga	配列番号１	２７８９０～２７８９２
mlcE	tga	配列番号２	５７３０～５７３２
mlcR	tag	配列番号２	１９１５～１９１７

Key: 1 Gene
2 Translation termination codon
3 Sequence No. containing translation termination codon
4 Nucleotide No. of the translation termination codons in Sequence Nos. 1 and 2
5 Sequence No.

* The base sequences represented by Sequence Nos. 1 and 2 of the sequence table are completely /50

complementary with respect to each other

In addition, Table 9 presents the C-terminal amino acid residue of the polypeptide hypothesized to be encoded by each structural gene, the base sequence of the trinucleotides that encode the amino acid residues and the locations of the trinucleotides.

Table 9. C-terminal amino acid of the polypeptide encoded by each structural gene

(1) 遺伝子	(2) C末端アミノ酸残基	(3) 該アミノ酸をコードするトリヌクレオチドの塩基配列	(4) 該トリヌクレオチドの存在する配列番号＊	(5) 配列番号１又は２における該トリヌクレオチドのヌクレオチド番号
mlcA	ｱﾗﾆﾝ (6)	gcc	配列番号２	32720～32722
mlcB	ｾﾘﾝ (7)	agt	配列番号２	19837～19839
mlcC	ｼｽﾃｲﾝ (8)	tgc	配列番号１	13476～13478
mlcD	ｱﾙｷﾞﾆﾝ (9)	cgc	配列番号１	27887～27889
mlcE	ｱﾗﾆﾝ (6)	gct	配列番号２	5727～5729
mlcR	ｱﾗﾆﾝ (6)	gct	配列番号２	1912～1914

Key:
1 Gene
2 C-terminal amino acid residue
3 Base sequence of the trinucleotide encoding the amino acid
4 Sequence No. in which the trinucleotide is present*
5 Nucleotide No. of the trinucleotide in Sequence No. 1 or 2
6 Alanine
7 Serine
8 Cysteine
9 Arginine
10 Sequence No.

* The base sequences represented by Sequence Nos. 1 and 2 of the sequence table are completely complementary to each other.

In addition, Table 10 presents the complementary sequence with respect to the translation termination codons of Table 8, the Sequence Nos. containing the complementary sequences, and the positions of the complementary sequences.

Table 10. Complementary sequences with respect to the translation termination codons in each of the structural genes.

1 遺伝子	2 翻訳終始コドンに対する相補配列	3 該相補配列の存在する配列番号＊	4 配列番号1又は2における該相補配列のヌクレオチド番号
mlcA	cta	5 配列番号1	1479～1481
mlcB	tta	配列番号1	14362～14364
mlcC	tta	配列番号2	20723～20725
mlcD	tca	配列番号2	6312～6314
mlcE	tca	配列番号1	28472～28474
mlcR	cta	配列番号1	32287～32289

Key: 1 Gene
2 Sequence complementary to the translation termination codon
3 Sequence No. containing the complementary sequence
4 Nucleotide No. of the complementary sequence in Sequence No. 1 or 2
5 Sequence No.

* The base sequence represented by Sequence Nos. 1 and 2 of the sequence table are completely complementary to each other. /51

As stated above, the presence of each structural gene, as well as the orientations and locations thereof, were elucidated, and based on these data it was possible to obtain the transcription product and translation product for each of the structural genes.

<u>Application example 9. Transformation of *Penicillium citrinum* using recombinant DNA vector pML48</u>

Transformation of *Penicillium citrinum* was carried out according to the method of Nara et al. (Nara, V., et al., Curr. Genet. 23, 28 (1993)).

1) Preparation of protoplasts

PGA agar medium was inoculated using a platinum loop from a slant produced by culturing *Penicillium citrinum* strain SANK 13380, and a temperature of 26°C was maintained

for 14 h. Spores of *Penicillium citrinum* strain SANK 13380 were recovered from the culture, and 10^8 spores were used in order to inoculate 80 mL of YPL-20 medium, whereupon a temperature of 26°C was maintained for 1 day. After confirming spore germination by means of microscopy, the germinated spores were recovered as sediment by centrifuging for 10 min under conditions of room temperature and 5000 xg. The spores were then washed three times with sterile water and were converted to protoplasts. Specifically, 200 mg of zymolyase 20T (Seikagaku Kogyo) and 100 mg of chitinase (Sigma) were dissolved in 10 mL of 0.55 M /52
magnesium chloride, and the supernatant obtained after centrifuging for 10 min under conditions of room temperature and 5000 xg was used as enzyme solution. 20 mL of enzyme solution and 0.5 g, wet weight, of germinated spores were introduced into a 100-mL Erlenmeyer flask, and the spores were gently agitated for 60 min at 30°C. Conversion of the germinated spores to protoplasts was confirmed by microscopy, and the reaction solution was then filtered with a 3G-2 glass filter (Hario). The filtrate was then centrifuged for 10 min under conditions of room temperature and 1000 xg and the protoplasts were recovered as sediment.

2) Transformation

The protoplasts obtained in (1) were washed twice with 30 mL of 0.55 M magnesium chloride solution, and once with 30 mL of 0.55 M magnesium chloride-50 mM calcium chloride-10 mM 3-morpholinopropanesulfonic acid (pH 6.3; "MCM solution" below), whereupon the material was suspended in 100 μL of 4% (w/v) polyethylene glycol 8000-10 mM 3-morpholinopropanesulfonic acid-0.0025% (w/v) heparin (Sigma)-50 mM magnesium chloride (pH 6.3; "transformation solution" below). 96 μL of transformation solution containing about 5 x 10^7 protoplasts and 10 μL of TE containing 120 μg of pML48 DNA were mixed and were left on ice for 30 min. To this was added 1.2 mL of 20% (w/v) polyethylene glycol-50 mM magnesium chloride-10 mM 3-morpholinopropanesulfonic acid (pH 6.3), and the solution was gently pipetted before leaving it for 20 min at room temperature. 10 mL of MCM solution were added and gently mixed, whereupon the solution was centrifuged for 10 min under conditions of room temperature and 1000 xg. The transformed protoplasts were then recovered from the sediment.

3) Regeneration of transformed protoplast cell walls

The transformed protoplasts obtained in (2) were suspended in 5 mL of liquid-form VGS middle-layer agar medium, and this material was layered over 10 mL VGS bottom-layer agar medium in a plate. The plate was then incubated at 26°C for 1 day, and 10 mL of liquid-form VGS top layer agar medium containing 5 mg of hygromycin B (Sigma) were then layered onto the plate (hygromycin B final concentration 200 μg/mL). The microorganism strain resulting /53

from maintaining a temperature of 26°C for 14 days was cultured for a generation on PGA agar medium containing 200 μg/mL hygromycin B, and was then transferred onto a slant produced using PGA agar medium, before maintaining a temperature of 26°C for 14 days.

These cells are referred to as "transformed strain".

The slant was stored at 4°C

Test Example 1. Comparison of ML-236B production capacity in transformed strain and original strain

The transformed strain obtained in Application Example 9 and original *Penicillium citrinum* strain SANK13380 were cultured and ML-236B production in the cultures was measured.

A slant on which the transformed strain had been cultured (refer to Application Example 9) or a slant on which *Penicillium citrinum* strain SANK13380 had been cultured (refer to Application Example 2) was used, and a 100 mL Erlenmeyer flask containing 10 mL of MBG3-8 medium was inoculated with 5 mm^2 of cells. After culturing for 2 days while shaking at 26°C, 3.5 mL of 50% (w/v) glycerin solution was added and culturing was continued for 10 days at 26°C while shaking.

50 mL of 0.2 N sodium hydroxide was added to 10 mL of the culture, and after maintaining a temperature of 26°C for 1 h while agitating, the material was centrifuged for 2 min under conditions of room temperature and 3000 xg. 1 mL of the supernatant was collected and was subjected to HPLC after mixing with 9 mL of 50% methanol.

SSC-ODS-262 (diameter 6 mm, length 100 mm; Senshu Kagaku) was used for the HPLC column, and the mobile phase was 75% (v/v) methanol-0.1% (v/v) triethylamine-0.1% (v/v) acetic acid. Elution was carried out at room temperature at a rate of 2 mL/min. The ML-236B eluted at 4.0 min after addition to the column under these conditions. Detection was carried out with the UV detector absorption wavelength set to 236 nm.

Five strains with improved ML-236B production capacity were obtained among the transformed strains, and these production capacities were increased by an average of 12% relative to the original strain.

The ML-236B production capacities of these 5 strains were stably maintained, even after passaging by a single spore treatment or the like.

Industrial Application field /54

DNA of the present invention obtained from an ML-236B-producing microorganism improves ML-236B production capacity when introduced into microorganisms that produce the compound.

The presence, orientation and location of each of the six structural genes on the DNA was elucidated. By means of the present invention, it is possible to obtain cDNA corresponding to each of the structural genes.

<u>Claims</u> /55

1. DNA characterized by comprising a base sequence represented by nucleotides 1-34203 of Sequence No. 1 of the sequence table, and by improving ML-236B biosynthesis in ML-236B-producing microorganisms when introduced into said microorganisms;

2. The DNA recited in Claim (1), which can be obtained from transformed *E. coli* strain pML48 SANK71199 (FERM BP-6780);

3. DNA characterized by hybridizing with the DNA recited in Claim (1) or (2), and by improving ML-236B biosynthesis in ML-236B-producing microorganisms when introduced into said microorganisms;

4. DNA characterized by hybridizing under stringent conditions with the DNA recited in Claim (1) or (2), and by improving ML-236B biosynthesis in ML-236B-producing microorganisms when introduced into said microorganisms;

5. Recombinant DNA vector comprising DNA recited in any one of Claims (1)-(4);

6. The recombinant DNA vector recited in Claim (5) which is carried by transformed *E. coli* strain pML48 SANK71199 (FERM BP-6780).

7. Host cells that have been transformed with the recombinant DNA vector recited in Claim (5) or (6).

8. The host cells recited in Claim (7) characterized by being an ML-236B-producing microorganism.

9. The host cells recited in Claim (8) characterized by being *Penicillium citrinum*.

10. A method for manufacturing ML-236B, characterized in that the host cells of Claim (8) or (9) are cultured, and ML-236B is then recovered from said culture.

11. The host cells according to (7), characterized by being *E. coli*.

12. The host cells according to (11) which are transformed *E. coli* strain ML48 SANK71199 (FERM BP-6780).

13. A PCR primer A1 having a sequence of at least 10 bases, where the 5'-terminus is the adenine of Nucleotide No. 23045 in Sequence No. 2 of the sequence table, or a base to the 5' side thereof.

14. A PCR primer A2 comprising a sequence comprising at least 10 bases and having at /56
least 70% homology with respect to the base sequence of the PCR primer A1 recited in Claim (13) (where said PCR primer A2 can be used in PCR in order to amplify cDNA encoding a

polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 23045 to 23047 of Sequence No. 2 of the sequence table).

15. A PCR primer A3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of the PCR primer A1 recited in Claim (13) (where said PCR primer A3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 23045 to 23047 of Sequence No. 2 of the sequence table).

16. A PCR primer A4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of the PCR primer A1 recited in Claim (13) (where said PCR primer A4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 23045 to 23047 of Sequence No. 2 of the sequence table).

17. A PCR primer B1 comprising a sequence comprising at least 10 bases having, as its 5'-terminus, the cytosine of Nucleotide No. 1479 in Sequence No. 1 of the sequence table or a base to the 5' side thereof.

18. A PCR primer B2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer B1 recited in Claim (17) (where said PCR primer B2 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the alanine residue encoded by Nucleotide Nos. 2720 to 32722 of Sequence No. 2 of the sequence table).

19. A PCR primer B3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer B1 recited in Claim (17) /57 (where said PCR primer B3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the alanine residue encoded by Nucleotide Nos. 32720 to 32722 of Sequence No. 2 of the sequence table).

20. A PCR primer B4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer B1 recited in Claim (17) (where said PCR primer B4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the alanine residue encoded by Nucleotide Nos. 2720 to 32722 of Sequence No. 2 of the sequence table).

21. A PCR primer C1 having a sequence comprising at least 10 based, where the 5'-terminus is the adenine of Nucleotide No. 11748 in Sequence No. 2 of the sequence table, or a base on the 5'-side thereof.

22. A PCR primer C2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer C1 recited in Claim (21) (where said PCR primer C2 can be used in PCR in order to amplify cDNA encoding a

polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 11748 to 11750 of Sequence No. 2 of the sequence table).

23. A PCR primer C3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer C1 recited in Claim (21) (where said PCR primer C3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 11748 to 11750 of Sequence No. 2 of the sequence table).

24. A PCR primer C4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer C1 recited in Claim (21) (where said PCR primer C4 can be used in PCR in order to amplify cDNA encoding a /58
polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 11748 to 11750 of Sequence No. 2 of the sequence table).

25. A PCR primer D1 having a sequence comprising at least 10 bases, where its 5'-terminus is the thymine of Nucleotide No. 14362 in Sequence No. 1 of the sequence table or a base on the 5'-side thereof.

26. A PCR primer D2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer D1 recited in Claim (25) (where said PCR primer D2 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the serine residue encoded by Nucleotide Nos. 19837 to 19839 of Sequence No. 2 of the sequence table).

27. A PCR primer D3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer D1 recited in Claim (25) (where said PCR primer D3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the serine residue encoded by Nucleotide Nos. 19837 to 19839 of Sequence No. 2 of the sequence table).

28. A PCR primer D4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer D1 recited in Claim (25) (where said PCR primer D4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the serine residue encoded by Nucleotide Nos. 19837 to 19839 of Sequence No. 2 of the sequence table).

29. A PCR primer E1 having a sequence comprising at least 10 bases, wherein its 5'-terminus is the adenine of Nucleotide No. 11796 in Sequence No. 1 of the sequence table or a base on the 5'-side thereof.

30. A PCR primer E2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer E1 recited in Claim (29) (where said PCR primer E2 can be used in PCR in order to amplify cDNA encoding a /59

polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 11796 to 11798 of Sequence No. 1 of the sequence table).

31. A PCR primer E3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer E1 recited in Claim (29) (where said PCR primer E3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 11796 to 11798 of Sequence No. 1 of the sequence table).

32. A PCR primer E4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer E1 recited in Claim (29) (where said PCR primer E4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 11796 to 11798 of Sequence No. 1 of the sequence table).

33. A PCR primer F1 having a sequence comprising at least 10 bases wherein its 5'-terminus is the thymine of Nucleotide No. 20723 in Sequence No. 2 of the sequence table or a base on the 5'-side thereof.

34. A PCR primer F2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer F1 recited in Claim (33) (where said PCR primer F2 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the cysteine residue encoded by Nucleotide Nos. 13476 to 13478 of Sequence No. 1 of the sequence table).

35. A PCR primer F3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer F1 recited in Claim (33) (where said PCR primer F3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the cysteine residue encoded by Nucleotide Nos. 13476 to /60
13478 of Sequence No. 1 of the sequence table).

36. A PCR primer F4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer F1 recited in Claim (33) (where said PCR primer F4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the cysteine residue encoded by Nucleotide Nos. 13476 to 13478 of Sequence No. 1 of the sequence table).

37. A PCR primer G1 having a sequence comprising at least 10 bases wherein its 5'-terminus is the adenine of Nucleotide No. 24321 in Sequence No. 1 of the sequence table or a base on the 5'-side thereof.

38. A PCR primer G2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer G1 recited in Claim (37) (where said PCR primer G2 can be used in PCR in order to amplify cDNA encoding a

polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 24321 to 24323 of Sequence No. 1 of the sequence table).

39. A PCR primer G3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer G1 recited in Claim (37) (where said PCR primer G3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 24321 to 24323 of Sequence No. 1 of the sequence table).

40. A PCR primer G4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer G1 recited in Claim (37) (where said PCR primer G4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 24321 to 24323 of Sequence No. 1 of the sequence table).

41. A PCR primer H1 having a sequence comprising at least 10 bases having, as its 5'-terminus, the thymine of Nucleotide No. 6312 in Sequence No. 2 of the sequence table or a base /61 on the 5'-side thereof.

42. A PCR primer H2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer H1 recited in Claim (41) (where said PCR primer H2 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the arginine residue encoded by Nucleotide Nos. 27887 to 27889 of Sequence No. 1 of the sequence table).

43. A PCR primer H3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer H1 recited in Claim (41) (where said PCR primer H3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the arginine residue encoded by Nucleotide Nos. 27887 to 27889 of Sequence No. 1 of the sequence table).

44. A PCR primer H4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer H1 recited in Claim (41) (where said PCR primer H4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the arginine residue encoded by Nucleotide Nos. 27887 to 27889 of Sequence No. 1 of the sequence table).

45. A PCR primer I1 having a sequence comprising at least 10 bases wherein its 5'-terminus is the adenine of Nucleotide No. 3545 in Sequence No. 2 of the sequence table or a base on the 5'-side thereof.

46. A PCR primer I2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer I1 recited in Claim (45) (where said PCR primer I2 can be used in PCR in order to amplify cDNA encoding a

polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 3545 to 3547 of Sequence No. 2 of the sequence table).

47. A PCR primer I3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer I1 recited in Claim (45) (where said PCR primer I3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 3545 /62 to 3547 of Sequence No. 2 of the sequence table).

48. A PCR primer I4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer I1 recited in Claim (45) (where said PCR primer I4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 3545 to 3547 of Sequence No. 2 of the sequence table).

49. A PCR primer J1 having a sequence comprising at least 10 bases wherein its 5'-terminus is the thymine of Nucleotide No. 28472 in Sequence No. 1 of the sequence table or a base on the 5'-side thereof.

50. A PCR primer J2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer J1 recited in Claim (49) (where said PCR primer J2 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the alanine residue encoded by Nucleotide Nos. 5727 to 5729 of Sequence No. 2 of the sequence table).

51. A PCR primer J3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer J1 recited in Claim (49) (where said PCR primer J3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the alanine residue encoded by Nucleotide Nos. 5727 to 5729 of Sequence No. 2 of the sequence table).

52. A PCR primer J4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer J1 recited in Claim (49) (where said PCR primer J4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the alanine residue encoded by Nucleotide Nos. 5727 to 5729 of Sequence No. 2 of the sequence table).

53. A PCR primer K1 having a sequence comprising at least 10 bases wherein its 5'-terminus is the adenine of Nucleotide No. 400 in Sequence No. 2 of the sequence table or a base on the 5'-side thereof.

54. A PCR primer K2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer K1 recited in Claim (53) /63

(where said PCR primer K2 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 400 to 402 of Sequence No. 2 of the sequence table).

55. A PCR primer K3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer K1 recited in Claim (53) (where said PCR primer K3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 400 to 402 of Sequence No. 2 of the sequence table).

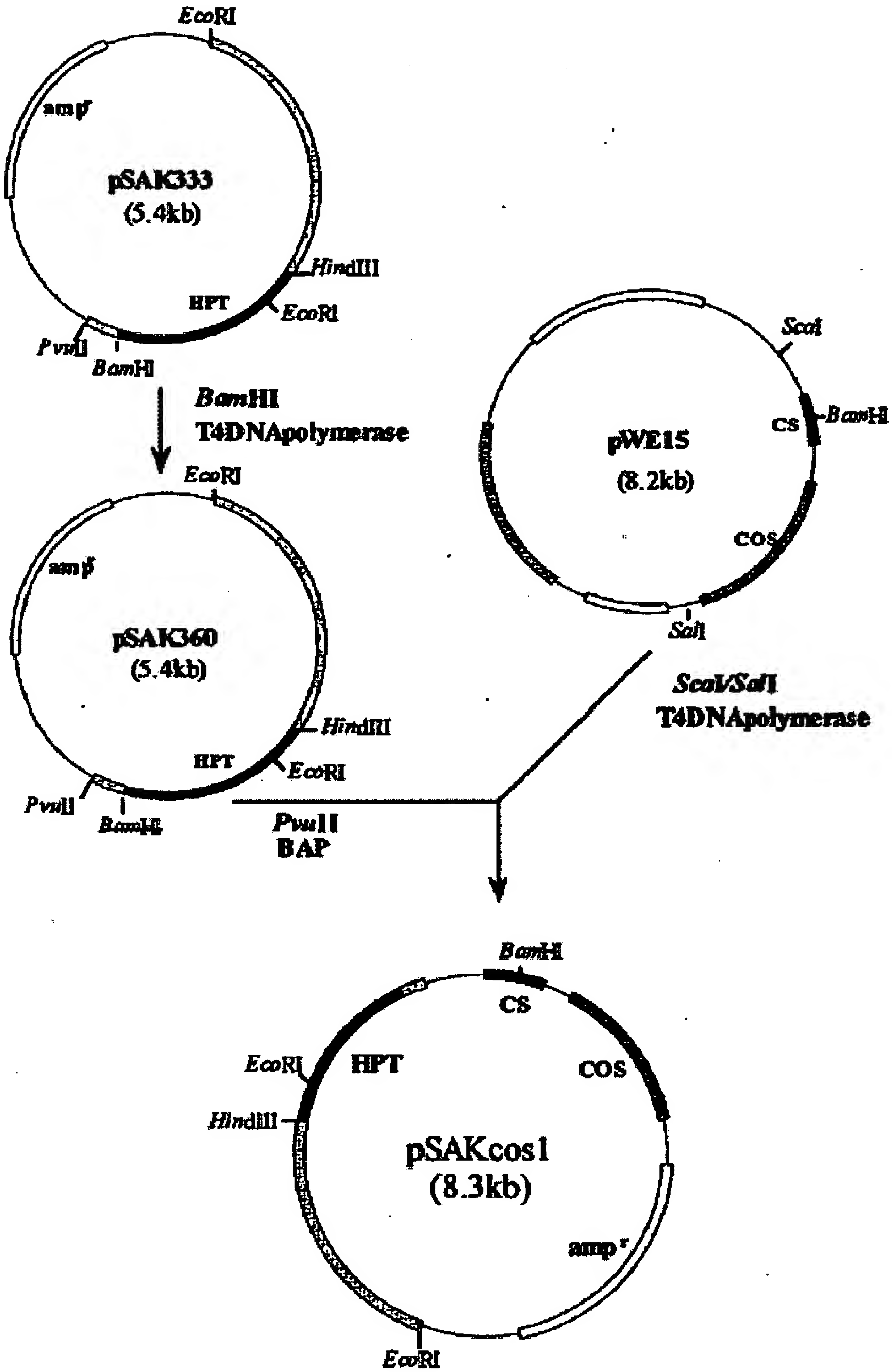
56. A PCR primer K4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer K1 recited in Claim (53) (where said PCR primer K4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its N-terminus, the methionine residue encoded by Nucleotide Nos. 400 to 402 of Sequence No. 2 of the sequence table).

57. A PCR primer L1 having a sequence comprising at least 10 bases wherein its 5'-terminus is the cytosine of Nucleotide No. 32287 in Sequence No. 1 of the sequence table or a base on the 5'-side thereof.

58. A PCR primer L2 comprising a sequence comprising at least 10 bases and having at least 70% homology with respect to the base sequence of PCR primer L1 recited in Claim (57) (where said PCR primer L2 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the alanine residue encoded by Nucleotide Nos. 1912 to 1914 of Sequence No. 2 of the sequence table).

59. A PCR primer L3 comprising a sequence comprising at least 10 bases and having at least 80% homology with respect to the base sequence of PCR primer L1 recited in Claim (57) (where said PCR primer L3 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the alanine residue encoded by Nucleotide Nos. 1912 to 1914 of Sequence No. 2 of the sequence table).

60. A PCR primer L4 comprising a sequence comprising at least 10 bases and having at least 90% homology with respect to the base sequence of PCR primer L1 recited in Claim (57) /64
(where said PCR primer L4 can be used in PCR in order to amplify cDNA encoding a polypeptide having, as its C-terminus, the alanine residue encoded by Nucleotide Nos. 1912 to 1914 of Sequence No. 2 of the sequence table).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ampʳ
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HPT
EcoRI
PvuII
BamHI
ScaI
CS
BamHI
pWE15
(8.2kb)
cos
SalI
ScaI/SalI
T4DNApolymerase
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ampʳ
EcoRI

Figure 1

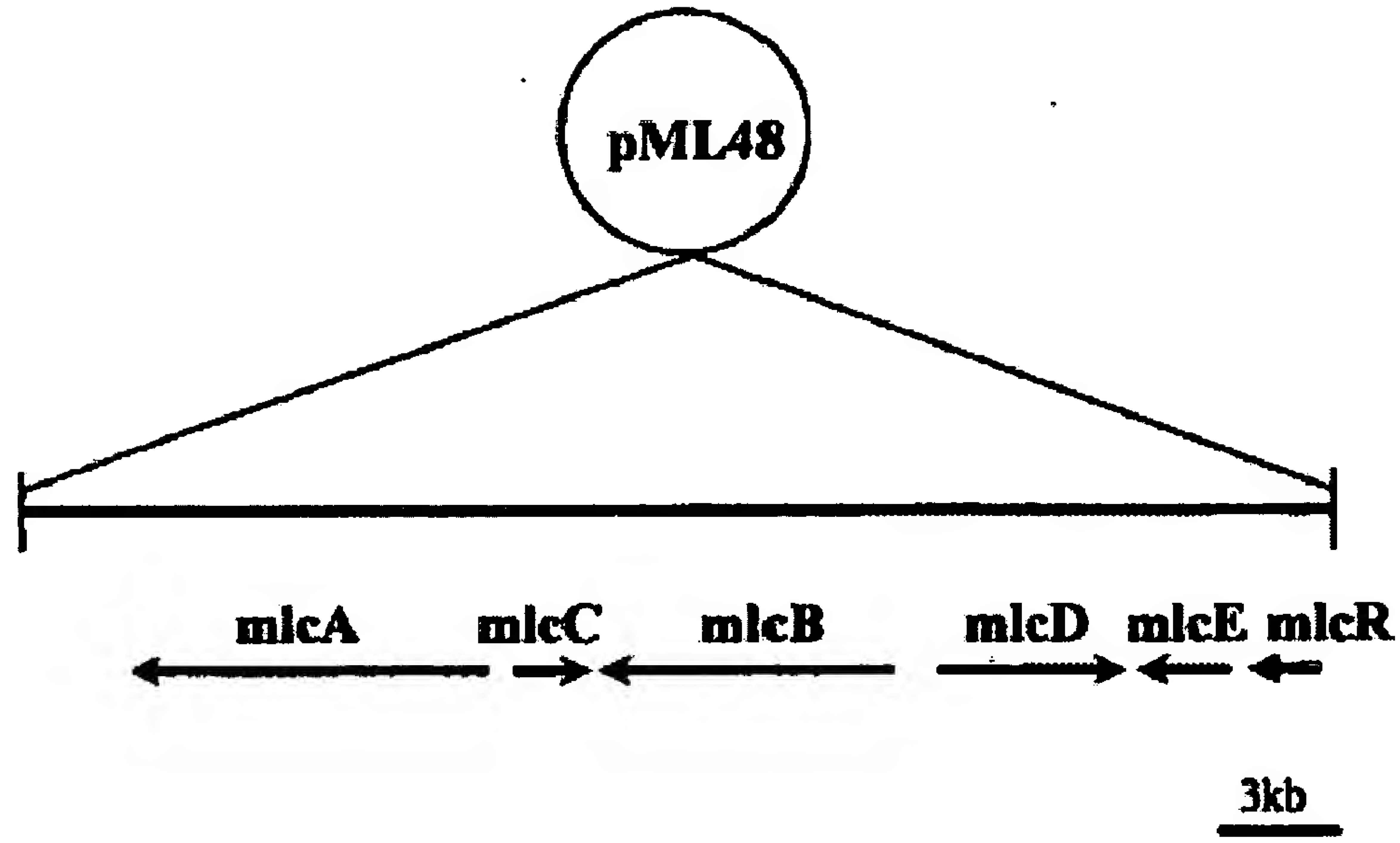

Figure 2. Position, size and orientation of the hypothesized structural genes (mlcA, mlcB, mlcC, mlcD, mlcE, mlcR) on the pML48 insertion sequence.

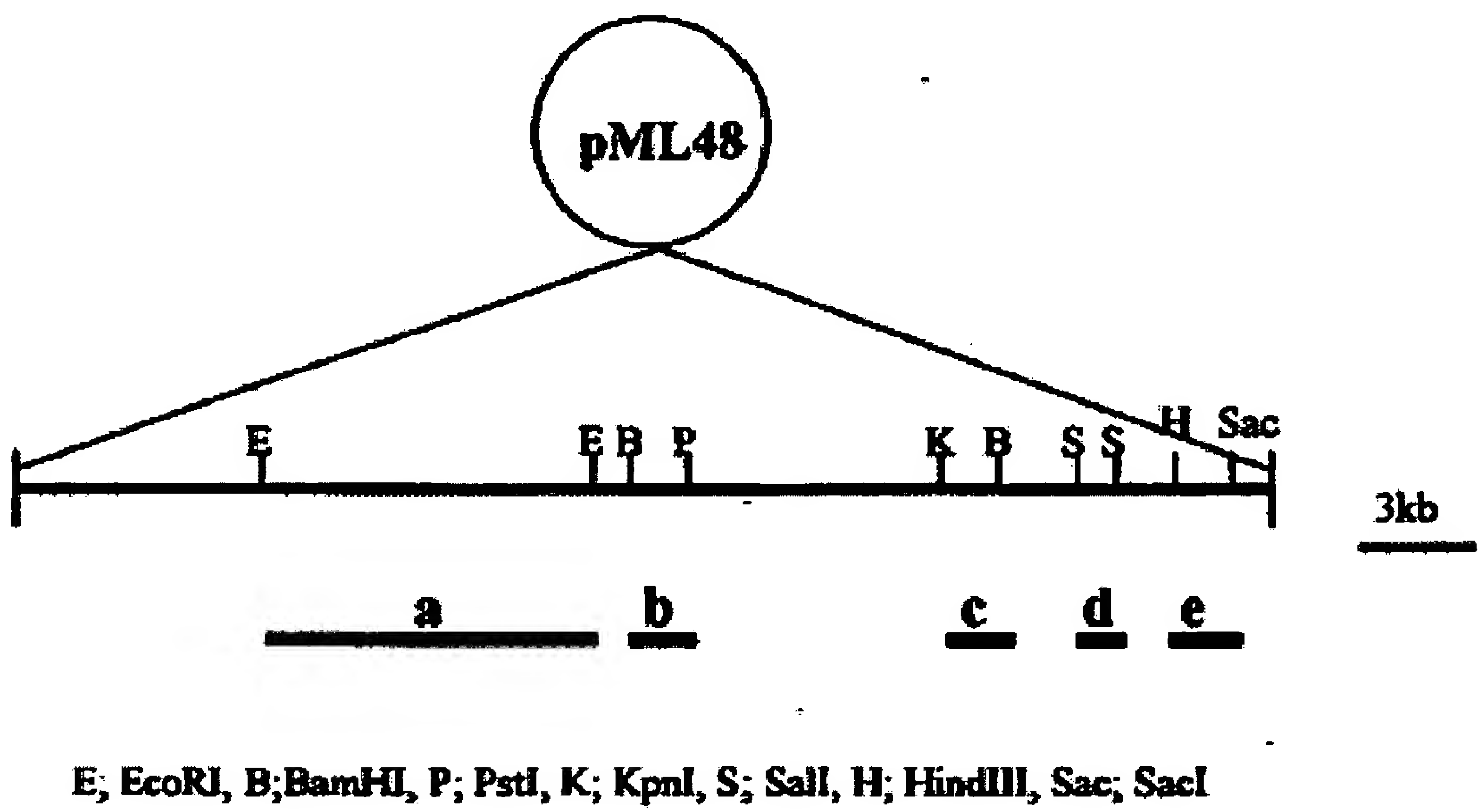

Figure 3A. Position of probes (a, b, c, d, e) used in Northern blot hybridization

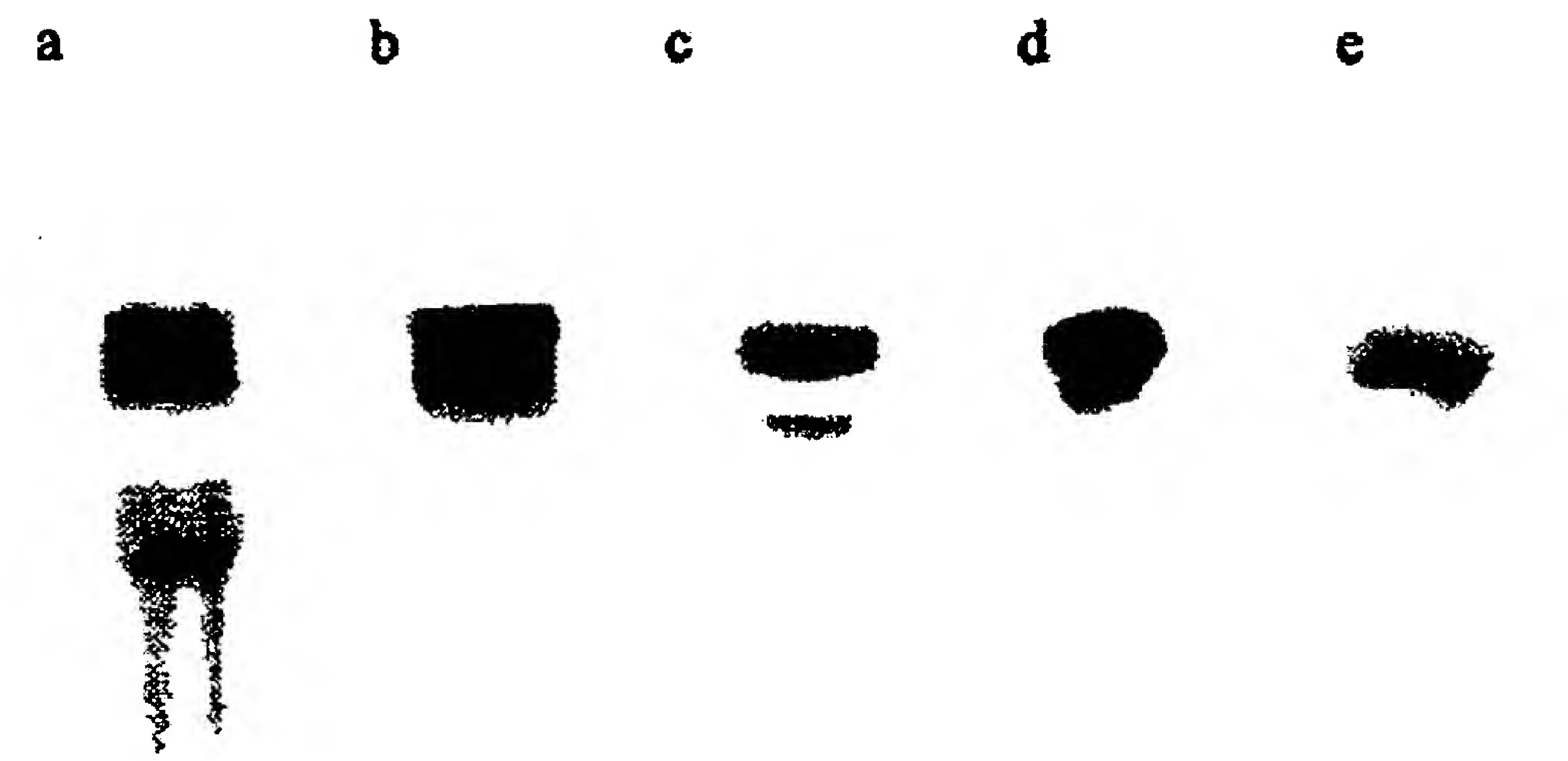

Figure 3B. Results of Southern blot hybridization